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Heart Specific *ScoX* Knockdown Induces *p53* Dependent Apoptosis and Cardiomyopathy

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Que Doña Leticia Martínez con D.N.I.: 16064390V, licenciada en Bioquímica ha realizado, bajo la dirección de los Directores de Tesis, en el Departamento de Bioquímica de la Facultad de Medicina de la Universidad Autónoma de Madrid / Instituto de Investigaciones Biomédicas "Alberto Sols", el trabajo titulado:

Heart Specific *ScoX* Knockdown Induces *p53* Dependent Apoptosis and Cardiomyopathy

Una vez supervisado el trabajo, consideramos que reúne todos los requisitos necesarios en cuanto a originalidad y calidad para ser presentado como Tesis Doctoral con el objeto de optar al título de Doctor en Ciencias por la Universidad Autónoma de Madrid.

Madrid, 7 de Mayo de 2014

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A mis padres y mi hermana

“ LOS CIENTÍFICOS DICEN QUE ESTAMOS HECHOS DE ÁTOMOS, PERO A MÍ UN PAJARITO ME CONTÓ QUE ESTAMOS HECHOS DE HISTORIAS”

EDUARDO GALEANO


HOW DO I LOVE YOU, THESIS?
LET ME COUNT THE WAYS...
I THINK ABOUT YOU NIGHT AND DAY.
I SEE YOU IN MY DREAMS.
???
I SING YOUR PRAISES FAR AND WIDE.
DATA
ZZZ
I WRITE ODES TO YOU NO ONE WILL EVER READ.
[REJECTED]
I WEEP AT YOUR ABSENCE.
I COUNT THE ~~DAYS~~ YEARS UNTIL YOU'RE MINE.
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RESUMEN/SUMMARY

La citocromo c oxidasa o complejo IV es el ultimo elemento de la cadena de transporte de electrones mitocondrial. Cataliza la oxidación del citocromo c transfiriendo sus electrones al oxígeno. El déficit de complejo IV debido a mutaciones en factores de ensamblaje es uno de los defectos más comunes de la cadena respiratoria. Estas patologías se caracterizan por su aparición a una edad temprana y cursar con un amplio espectro de manifestaciones clínicas como encefalopatía, cardiomiopatía, hepatopatía o leucodistrofia.

SCO1 y SCO2 son dos metalochaperonas responsables de la formación del centro de cobre Cu_A en el ensamblaje del complejo IV. Mutaciones en SCO1 causan hepato-encefalomiopatía aunque se ha descrito un caso que también presenta cardiomiopatía hipertrófica. Las mutaciones en SCO2 causan cardiomiopatía hipertrófica y encefalopatía infantil. A excepción de un caso, todos los pacientes portan la mutación E140K.

En esta tesis hemos utilizado *Drosophila melanogaster* como sistema modelo para el estudio de las bases genéticas y moleculares de la cardiopatía causada por mutaciones en las proteínas SCO. Los mecanismos genéticos que controlan la especificación de los cardiomiocitos y numerosos aspectos de la fisiología del corazón están conservados en *Drosophila* lo que hace de este organismo un excelente modelo para el estudio de la función cardiaca y las cardiomiopatías humanas. Además, recientemente se han desarrollado técnicas que permiten caracterizar la fisiología del corazón de *Drosophila*, facilitando el estudio de cómo defectos en la cadena de transporte de electrones, afectan a la función cardiaca.

Drosophila presenta un solo ortólogo para los genes de mamífero *Sco1* y *Sco2*, *ScoX*. Los resultados obtenidos demuestran que la interferencia de *ScoX* en el corazón causa una cardiomiopatía dilatada, viéndose gravemente afectada tanto la función como la estructura del corazón. Los cardiomiocitos sufren un cambio metabólico favoreciendo la glicólisis frente a la fosforilación oxidativa, causando, probablemente, acidosis láctica y emulando los síntomas clínicos observados en pacientes con mutaciones en *Sco1* y *Sco2*. El fenotipo observado es debido a una activación, dependiente de *dp53,* de la muerte celular. Estos resultados sugieren que dp53 contribuye directamente en el desarrollo de la cardiomiopatía.

Cytochrome c oxidase or complex IV is the terminal component of the mitochondrial electron transport chain. It catalyses the transfer of electrons from reduced cytochrome c to molecular oxygen. Complex IV deficiency due to mutations in assembly factors is one of the most frequent defects of respiratory chain in humans. These pathologies are characterized by a very early age of onset and the display of different clinical presentations, as encephalopathy, cardiomyopathy, hepatic failure and leukodystrophy.

SCO1 and SCO2 are two metallochaperones playing a key role in the formation of cooper centre Cu_A during complex IV assembly. Mutations in SCO1 cause hepato-encephalomyopathy although one case has been reported which also presents hypertrophic cardiomyopathy. Mutations in SCO2 cause hypertrophic cardiomyopathy and infantile encephalomyophathy and with but one exception, all patients harbour the E140K mutation.

In this thesis, we have used *Drosophila melanogaster* as model system to investigate the genetic and molecular mechanisms that underlie the cardiomyopathy associated with SCO deficiency. The genetic network controlling cardiac specification and differentiation as well as many aspects of heart function are conserved from flies to mammals. Thus, *Drosophila* has become a powerful model system for the study and understanding of cardiac function and human cardiomyopathies. Furthermore, the recently established heart function assays in *Drosophila* make it possible to characterize how mitochondrial electron transport chain defects affect heart function.

In *Drosophila* there is a single ortholog of mammalian *Sco1* and *2*, *ScoX*. Our findings demonstrate that cardiac-specific knockdown of *ScoX* causes dileted cardiomyopathy severely compromising heart function and structure. Cardiomyocytes undergo a metabolic switch from oxidative phosphorylation to glycolysis probably accompanied by lactic acidosis and therefore mimicking the clinical features found in patients with mutations in *Sco1* and *Sco2*. The observed phenotype is result of dp53-dependent cell death activation. These results strongly suggest that dp53 is directly involved in cardiomyopathy development.

INDEX

ABBREVIATIONS

$\Delta\mu H$	Electrochemical potencial
$\Delta\Psi$	Electrical gradient
AIF	Apoptosis-inducing factor
CI	Complex I
CII	Complex II
CIII	Complex III
CIV	Complex IV
CoQ	Coenzyme Q
COX	Cytochrome c oxidase
CsA	Cyclosprin A
CV	ATP synthase
CypD	Cyclophilin D
cyt c	Cytochrome c
DHE	Dihydroethidium
DI	Diastolic interval
dpp	Decapentaplegic

ETC	Electron Transport Chain
FS	Fractional Shortening
GPI	Glucose-6-phosphate isomerase
HFD	High fat diet
HK2	Hexokinase II
HP	Heart period
IMM	Inner mitochondrial membrane
IMS	intermembrane space
LDH	Lactate dehydrogenase
LS	Leigh Syndrome
MOMP	Mitochondrial outer membrane permeabilization
mPTP	Mitochondrial permeability transition pore
MRC	Mitochondrial respiratory chain
MRCD	Mitochondrial Respiratory Chain Disorders
mtDNA	Mitochondrial DNA
OMM	Outer mitochondrial membrane

OXPHOS	Oxidative phosphorylation
p53R2	p53-inducible ribonucleotide reductase
PDC	Pyruvate dehydrogenase complex
PDH	Pyruvate dehydrogenase
PDK2	Pyruvate dehydrogenase kinase 2
PDK	Pyruvate dehydrogenase kinase
PFK	Phosphofructokinase
PGM	Phophoglycerate mutase
PGM-M	Muscle isoform of PGM
POLG	DNA polymerase γ
PPP	Pentose phosphate pathway
ROS	Reactive oxygen species
RPL0	Ribosomal protein L0
SDH	Succinate Dehydrogenase
TIGAR	TP53-induced glycolysis and apoptosis regulator
Tin	Tinman

INTRODUCTION

1.THE MITOCHONDRIA

In 1970, Lynn Margulis published Origin of Eukariotic Cells, where she postulated the endosymbiotic hypothesis about a prokaryotic origin for eukaryotic mitochondria (Lynn Margulis 1970). According to this theory, present eukaryotic cells originated from a beneficial symbiosis between two free-living cells. Indeed, a α-proteobacterium was supposedly taken inside the pre-eukaryotic host cell and then formed an obligate endosymbiont (Lynn Margulis 1970; Martin et al. 2001). The discovery of DNA within mitochondrion (mtDNA), together with the finding that they contain a translation system distinct from that of the cytosol, were two of the observations that Margulis marshalled in support of the endosymbiont hypothesis. Indeed, throughout her career,

Margulis forcefully argued that simbiosis is a potent but largely unrecognized and unappreciated force in evolution (L. Margulis and Bermudes 1985).

Mitochondria have always being termed the powerhouses of eukaryotic cells due to their central role in ATP production through a process called oxidative phosphorylation (OXPHOS) and carried out by the electron transport chain (ETC, see below). However, mitochondria play a very important role in cell metabolism. Not only it is responsible of energy production but it also has crucial roles in amino acid and lipid metabolism and the biosynthesis of heme group and iron-sulphur clusters (Schmidt et al. 2010). Mitochondria is a major site of reactive oxygen species (ROS) production (Starkov 2008) and a key player in apoptosis (Tait and Green 2010) and calcium homeostasis (Mammucari et al. 2011).

The mitochondrial OXPHOS machinery is essential for cell function, maintenance, and survival. In mammals, OXPHOS provides more than 90% of cellular energy from the oxidation of different substrates, mainly pyruvate, the product of glycolysis, and fatty acids. During the Krebs cycle and the β-oxidation of fatty acids electrons are transferred from the intermediates being oxidized to the hydrogen carriers NAD^+ and FAD^+ which, in turn, feed the reducing equivalents into the respiratory chain in the inner mitochondrial membrane (Drose and Brandt 2012).

One of the consequences of oxidative phosphorylation is the generation of mitochondrial ROS. Mitochondria constitute a major source of ROS within cells (Murphy 2009). At high concentrations, ROS can damage lipids, proteins and DNA, and can induce cell death (Spierings et al. 2005). But even if ROS have been mostly associated with oxidative damage and pathology, they can also function as regulatory molecules or "second messengers", thus playing an important role in normal cell function and physiology (Murphy et al. 2011).

Mitochondria are highly dynamic organelles, which ultrastructure, morphology and distribution varies enormously among cell types. Mitochondria are often organized in the cytoplasm as a network, a reticulum of interconnected organelles shaped by fusion and fission events (Bereiter-Hahn and Voth 1994; Scorrano 2013). Its cytosolic localization is not random; these organelles accumulate where high amounts of ATP are required or where Ca2+ signalling needs to be tightly regulated. Thanks to the continuous studies in the field, we have learned that changes in mitochondrial shape influence crucial cellular functions, from Ca2+ signalling to ROS generation, neuronal plasticity, muscle atrophy, lymphocyte migration and even lifespan (Campello and

Scorrano 2010).

Mitochondrial malfunction causes a wide range of syndromes associated with metabolic and degenerative diseases as well as cancer and aging (Wallace 2007). They can have its origin in mutations in mtDNA or in nuclear-encoded mitochondrial genes (Koopman et al. 2012; Schapira 2006). The currently accepted unifying point of mitochondrial disease is that mutations in either mtDNA or nDNA mitochondrial genes lead to a decreased respiratory chain/OXPHOS performance thus, causing diseases associated to energy deficiency (Smeitink et al. 2006).

Mitochondrial disorders due to OXPHOS dysfunction are one of the most frequent inborn errors of metabolism, with an incidence of 1:5000 live births (Schaefer et al. 2004; Skladal et al. 2003). They can be caused by mutations in the nuclear or the mitochondrial genome and are characterized for being associated with a broad spectrum of clinical presentations. They might affect single or multiple organs, particularly those with a high energy demand such as brain, heart, skeletal muscle, liver, and endocrine systems (Schapira 2006). Cardiac involvement is a common feature associated with Mitochondrial Respiratory Chain Disorders (MRCD), being neonatal manifestation the most prevailing feature. When MRCD affects the heart may cause Dilated (with abnormal chamber size) or Hypertrophic (with abnormal wall thickness) cardiomyopathies. Neonatal cardiac abnormalities can be either isolated or accompanied with multi-organ involvement being frequently associated to metabolic crises and lactic acidosis, having fatal outcome (Schiff et al. 2011).

Although many mutations have been found to be responsible for OXPHOS defects, their pathogenic mechanisms are still poorly understood. In the last decade, numerous animal models ranging from invertebrates to mammals have been developed in order to understand their pathophysiology. In most cases, models mimic the pathological features observed in humans and are paving the way for the development of new alternative treatments for mitochondrial diseases.

1.1. Mitochondrial structure

Mitochondria have a structure distinct from that of other organelles since they contain two membranes: the outer mitochondrial membrane (OMM) and the inner mitochondrial membrane (IMM), which separates the intermembrane space (IMS) from the matrix. The IMM is the active site for the electron transport chain and ATP production, being its integrity crucial for mitochondrial function (Schenkel and Bakovic 2014). The

IMM is highly folded and protrudes into the matrix by invaginations called cristae. In consequence, the IMM has a large surface area that increases the efficiency of the chemical reactions occurring at its inner surface (Mannella 2008).

1.2. Mitochondrial genetic system

mtDNA consist in a double stranded circular molecule ranging in size between 16 and 21 kb which is replicated and transcribed within the organelle. It encodes 22 tRNAs and 2 rRNAs (12S and 16S), plus 13 subunits of the OXPHOS system: seven subunits of the NADH: Ubiquinone oxidoreductase (Complex I), one subunit of the Ubiquinone: Cytochrome c oxidoreductase (Complex III), three subunits of the Cytochrome c Oxidase (Complex IV) and two subunits of the H1-ATP synthase (Complex V) (Figure 1). The rest of the OXPHOS subunits and all of the proteins involved in the mtDNA transcription and translation are encoded in the nucleus. Therefore, OXPHOS complexes synthesis depends on the coordinated expression of both mitochondrial and nuclear genomes (Diaz and Moraes 2008).

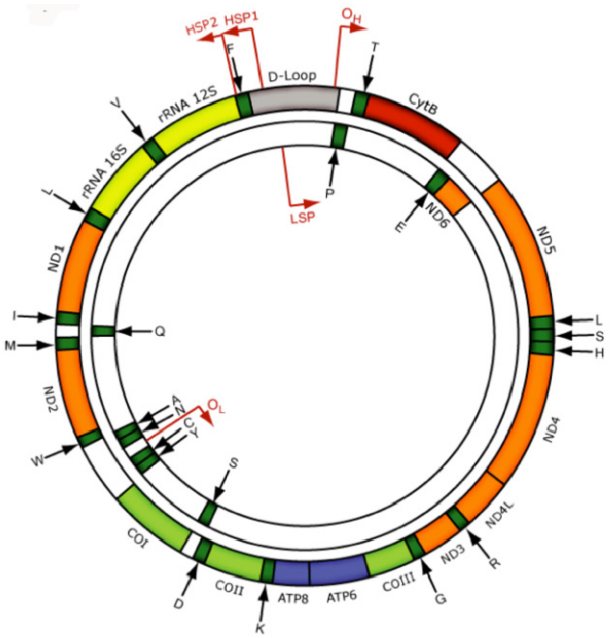

Figure 1. Schematic representation of human mtDNA. Protein and rRNA coding genes are represented by colored boxes. tRNA genes, dark green boxes, are identified by the single letter code. The regulatory elements located in the D-loop are indicated. O_H and O_L origin of replication of the heavy and light strands, HSP and LSP heavy and light strand promoters.

The mitochondrial genome is maternally inherited; each mitochondrion contains

several copies of mtDNA (Shuster et al. 1988). In a given individual, all mtDNA copies are thought to be identical, a condition known as homoplasmy. Mutations can arise, be maintained or amplified to different levels and coexist with wild-type mtDNA, giving rise to heteroplasmy (Lightowlers et al. 1997). In consequence, it is common to find a threshold effect in mtDNA-linked human diseases; the number of mutated molecules has to reach a certain percentage, usually higher than 60-80%, in order to manifest pathological effects (Fernandez-Silva et al. 2003).

2. MITOCHONDRIAL ENERGETICS: The mitochondrial respiratory chain

The MRC is composed of five multiheteromeric complexes, complexes I to V: CI, CII, CIII, CIV or cytochrome c oxidase (COX) and CV or ATP synthase, embedded in the IMM, and two mobile electron shuttles, Coenzyme Q (CoQ) and cytochrome c (cyt c) (Figure 2). Electrons enter the ETC mainly through CI from NADH. In addition, CII feeds electrons to CoQ derived from succinate, linking the Krebs cycle with OXPHOS. CoQ supplies these electrons to CIII that transfers one electron at a time to cyt c, which passes it to COX. COX eventually fixes four electrons to molecular oxygen to form two water molecules. This process, known as respiration, liberates energy that is partly converted by the proton pumping activity of CI, CIII and CIV into an electrochemical potential ($\Delta\mu H$) composed of an electrical gradient ($\Delta\Psi$) and a pH gradient, across the IMM. $\Delta\mu H$ constitutes the driving force not only for the phosphorylation of ADP to ATP by CV, but also for a number of other processes, such as heat production, Ca^{2+} import inside mitochondria and protein translocation (Ghezzi and Zeviani 2012).

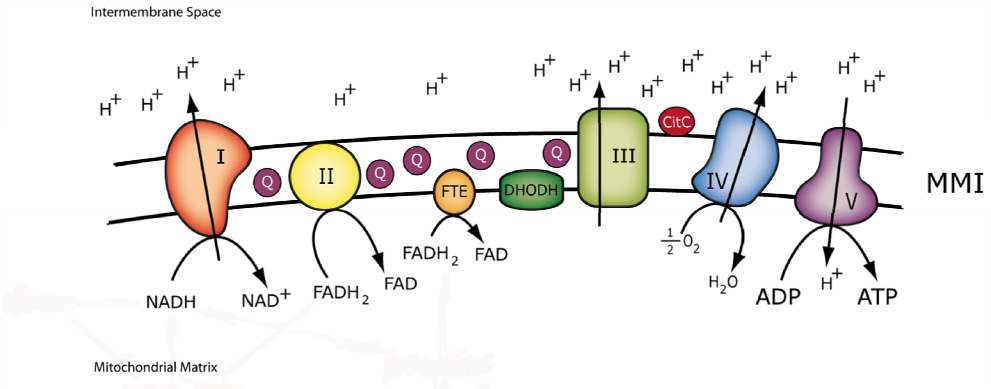

Figure 2. Schematic diagram of mitochondrial electron transport chain complexes. NADH and $FADH_2$ donate electrons to protein complexes I and II, which transferred through the ETC to CIV. The transfer of electrons yields energy that is used to pump protons to the intermembrane space. This leads to the establishment of electrochemical potential ($\Delta\mu H$). The $\Delta\mu H$ is used by complex V to drive synthesis of ATP.

3. CYTOCROME C OXIDASE

3.1. Structure of Cytochrome c Oxidase

Cytochrome c oxidase is the terminal component of MRC. It catalyzes the transfer of electrons from reduced cyt c to molecular oxygen. Mammalian COX is a 200 kDa multimeric protein complex comprised of 13 structural subunits, embedded in the IMM, and being active as a dimer. The enzyme core is composed by the three mitochondrially encoded subunits (CO I-III) (Tsukihara et al. 1996). It has two copper binding sites (Cu_A and Cu_B), two hemes groups found exclusively in COX (*a* and a_3), and magnesium and zinc ions (Tsukihara et al. 1996; Yoshikawa et al. 1996). Both heme *a* and the binuclear center a_3-Cu_B are located in COX1 whereas the binuclear Cu_A centre is located in COX2. A Zn^{2+} ion is in COX5b on the matrix side of the complex. The Mg^{2+} ion is close to the a_3-Cu_A site, between COX1 and COX2. Complex IV can also bind calcium and sodium ions (Tsukihara et al. 1995, 1996).

3.2. Assembly of Cytochrome c Oxidase

The assembly of individual structural subunits into a fully functional holoenzyme is a rather complicated process, with the formation of three distinct assembly intermediates, S1-S3, preceding that of the mature holoenzyme and which requires nuclear-encoded accessory factors (Leary 2010; Nijtmans et al. 1998). The assembly starts with the insertion of newly synthesized COX1 into the inner mitochondrial membrane. This first, crucial, step (S1) is followed by the incorporation of subunits COX4 and COX5a, to form a second assembly intermediate, S2. Insertion of heme *a* is likely to occur just after the formation of S1 or during the formation of S2, together with the insertion of Cu_B and heme a_3 into COX1 (Antonicka et al. 2003b; Antonicka et al. 2003a; S. L. Williams et al. 2004). The formation of the COX2-associated CuA center is followed by the incorporation of COX2 into the S2 intermediate (Figure 3).

Next, rapid sequential incorporation of COX3 and the smaller nuclear encoded subunits, COX5b and COX8, to form S3 leads to the formation of a quasi-complete assembly intermediate (subcomplex b) (Massa et al. 2008; Stiburek et al. 2005; S. L. Williams et al. 2004). The addition of the remaining subunits, including COX6a, COX6b, COX7a and COX7b, results in the formation of holocomplex monomer (S4) (Figure 3) (Massa et al. 2008; Nijtmans et al. 1998). Finally, monomeric COX dimerizes in an active structure containing the cyt c binding site (Tsukihara et al. 1996).

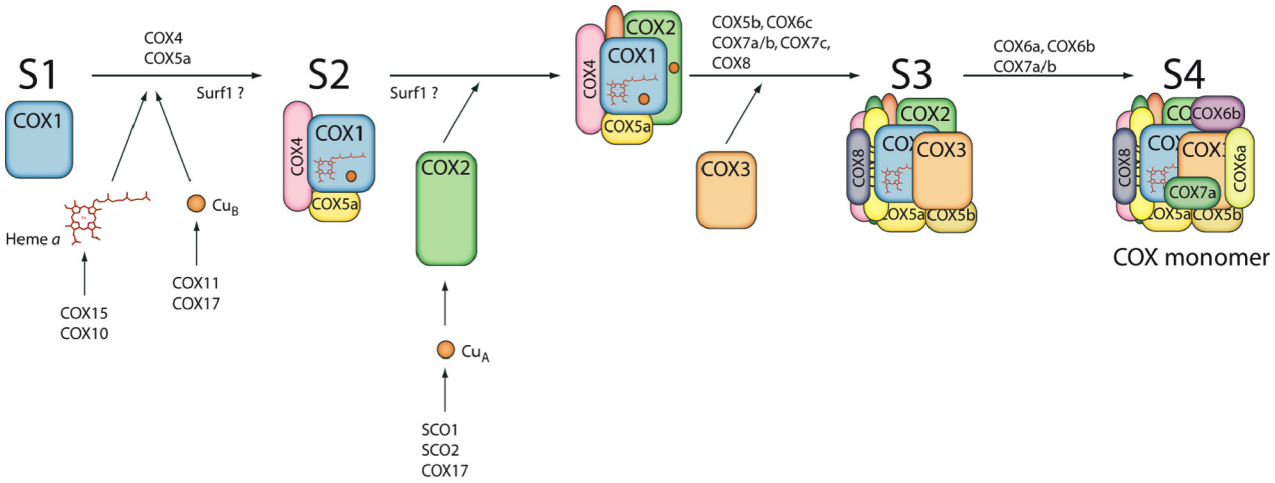

Figure 3. Schematic outline of CIV assembly and assembly factors. The formation of three distinct assembly intermediates, S1-S3, are represented as is the fully assembly holocomplex monomer (S4.)

3.2.1 Cytochrome c oxidase assembly complexes

The assembly of COX holoprotein requires numerous nuclear-encoded factors, including those responsible for the synthesis of heme *a* (COX10 and COX15) and the transport and insertion of copper ions (SCO1 and SCO2). In yeast, more than 30 chaperones assist COX assembly, many of them having human homologues, including CMC1, COX10, COX11, COX15, COX17, COX18, COX19, COX20, LRPPRC, OXA1, PET191, SCO1, SCO2, and SURF1 (Yang et al. 2010).

SURF1 is a 30 kDa protein that participates in the formation of the early COX subcomplexes. Although the mechanistic function of SURF1 protein is still unclear, the accumulation of S1 and S2 subassemblies in human mutant cells indicates a role for this protein in the early stages of CIV assembly, most likely before the incorporation of COX2 into the S2 assembly intermediate (Nijtmans et al. 1998; Stiburek et al. 2005; Tiranti et al. 1999). Recent studies in bacteria have indicated that prokaryotic SURF1 orthologs, Surf1c and Surf1q, are heme *a* binding proteins, suggesting a role for SURF1 in the insertion of heme *a* into the a_3 center or in the stabilization of the a_3-Cu_B binuclear center into COX1 (Bundschuh et al. 2009).

COX10 and COX15 are involved in the terminal steps of hemes *a* and *a3* biosynthesis (Barros et al. 2001; Barros et al. 2002; Barros and Tzagoloff 2002), while

COX19 is a cytosolic protein with a similar structure to that of COX17, suggesting a role in copper translocation to mitochondria (Ghezzi and Zeviani 2012).

SCO1 and SCO2 are paralog genes encoding two metallochaperones playing a role in the formation of the Cu_A site on COX2. They harbour a highly conserved Cx_3C domain that is thought to bind cooper (Horng et al. 2005). SCO2 synthesis is transcriptionally activated by p53, which modulates the balance between OXPHOS and glycolysis (Matoba et al. 2006). Both SCO proteins are essential for the assembly of complex IV catalitic core and play a role in the maintenance of cellular copper homeostasis (Leary et al. 2007).

The incorporation of cooper into COXII requires additional factors, partly conserved in yeast and mammals, including COX17 and COX11. At the early steps of COX assembly, COX17 donates $Cu2^+$ to SCO proteins and COX11 for the biogenesis of CuA and CuB sites (Banci et al. 2008; Cobine et al. 2006; Horng et al. 2004). While a single SCO protein, SCO1, is necessary for CuA site formation in yeast (Dickinson et al. 2000; Glerum et al. 1996), both SCO proteins are required in humans for the synthesis, maturation, and insertion of COXII into the nascent holoenzyme complex (Jaksch et al. 2000; Leary et al. 2009b; Papadopoulou et al. 1999; Valnot et al. 2000).

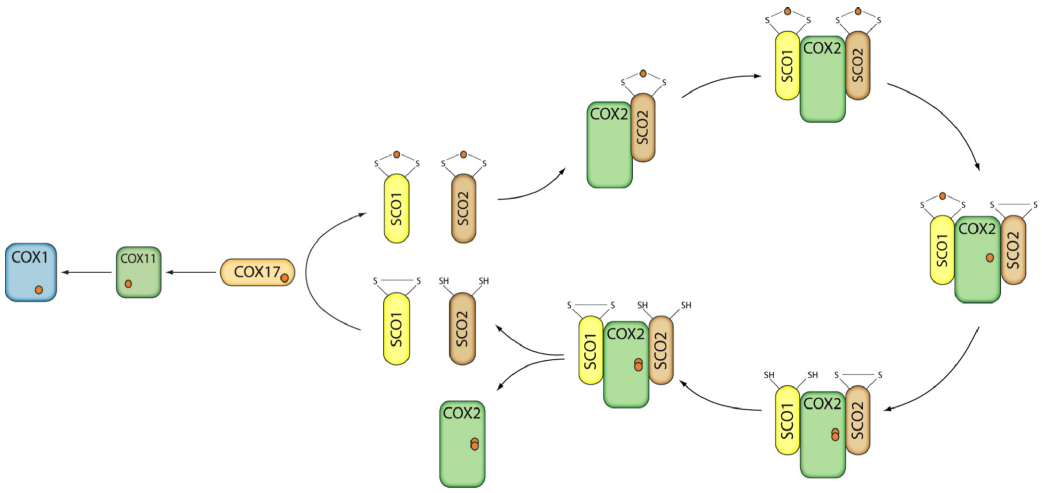

Figure 4. Schematic illustration of mitochondrial copper trafficking pathways. COX17, transfers copper to COX11 for maturation of the CuB site of COX I, or to SCO1 and SCO2 for maturation of the CuA site of COX2. Metallation of COX1 and COX2 allows the progression through the early stages of COX assembly and maturation of the fully assembled holoenzyme (COX).

Despite the requirement of both SCO proteins in humans, studies in SCO1 and SCO2 patient derived cell lines have demonstrated that SCO proteins have non-overlapping but cooperative roles in Cu_A biogenesis (Leary et al. 2004). Human SCO2 interacts with newly synthesized COX2, as it is inserted into the inner membrane or immediately thereafter, an association that depends on its prior metallation by COX17 (Figure 4). The physical interaction between SCO2 and COX2 triggers the metallation of SCO1 by COX17 and its recruitment to the SCO2-COX17 complex. Each SCO protein sequentially delivers a copper ion to COX2 to form the Cu_A site. The mature polypeptide is then incorporated into the nascent holoenzyme. Initially, SCO2 donates its copper with its cysteine thiols becoming oxidazed in the process. After the subsequent transfer of copper from SCO1 to COX2, SCO2 acts as a thiol-disulfide oxidoreductase to reoxidize the cysteines in SCO1. Alternatively, SCO2 may induce disulfide bond formation in copper-loaded SCO1 to facilitate its transfer to COXII (Figure 4) (Leary et al. 2004; Leary et al. 2009b).

3.3. Cytochrome c Oxidase Deficiency

Cytochrome c oxidase deficiencies are one of the most common defects of the respiratory chain found in mitochondrial diseases. Just one pathogenic mutation has been reported in a nuclear-encoded structural CIV subunit, COX6B1, in two cases of mitochondrial encephalomyopathy with isolated COX deficiency (Massa et al. 2008).

In humans, most syndromes with isolated complex IV deficiency are caused by mutations in genes encoding COX assembly factors that affect the stability and incorporation of COX subunits into the assembled complex such as SURF1 (Pequignot et al. 2001; Tiranti et al. 1998; Zhu et al. 1998), SCO1 and SCO2 (Leary et al. 2004; Papadopoulou et al. 1999; Valnot et al. 2000), COX15 (Antonicka et al. 2003a; Bugiani et al. 2005; Oquendo 2004), COX10 (Antonicka et al. 2003b; Coenen et al. 2004) and LRPPRC (Mootha et al. 2003; Xu et al. 2004). They are associated with different clinical presentations, including encephalopathies, Leigh Syndrome (LS), hypertrophic cardiomyopathies, fatal lactic acidosis, hepatic failure and leukodystrophy.

More that 40 different pathogenic mutations have been reported in the *Surf1* gene, all causing LS associated with COX deficiency (Pecina et al. 2004; Pequignot et al. 2001). Almost all *Surf1* mutations reported to date cause the complete absence of the protein, a marked reduction in the amount of fully assembled COX and the accumulation of assembly intermediates S1 and S2 (Stiburek et al. 2005; Tiranti et al. 1999).

Mutations in *COX10* are associated with a wide spectrum of conditions including LS; encephalopathy with proximal renal tubulopathy; sensorineural deafness, metabolic acidosis, hypotonia and hypertrophic cardiomyopathy. Likewise, mutations in *COX15* gene can cause fatal infantile hypertrophic cardiomyopathy, as well as LS (Antonicka et al. 2003b; Antonicka et al. 2003a; Bugiani et al. 2005).

Pathogenic mutations in either *Sco1* or *Sco2* result in severe COX deficiency with different early onset and fatal clinical phenotypes. Mutations in *Sco1* cause fatal infantile hepato-encephalomyopathy (Leary et al. 2013) and one case has been reported with hypertrophic cardiomyopathy, encephalopathy and hepatomegaly with fatal outcome (Stiburek et al. 2009). Mutations in *Sco2* cause a fatal infantil cardio-encephalomyophathy and, but with one exception, all patients harboured the missense mutation E140K (Mobley et al. 2009; Papadopoulou et al. 1999).

Heart hypertrophy, which has a direct life-threatening impact, is usually severe in all *Sco2* mutant patients. Despite the similar functional involvement of SCO1 and SCO2 as copper-binding proteins, the precise molecular function of the two human SCO proteins in COX assembly remains unknown. Moreover, their ubiquitous expression with no strict tissue specificity for either protein arise the question of why mutations in the two genes lead to different tissue-specific deficiencies in COX with distinct clinical phenotype (Brosel et al. 2010).

5. DROSOPHILA MELANOGASTER

The common fruit fly, *Drosophila melanogaster*, is a well studied, highly tractable genetic model organism for the understanding of the molecular mechanisms underlying human diseases. *Drosophila melanogaster* have a short life cycle, approximately 10 days from egg lay to adult hatching from the puparium at 25ºC. The embryo completes its development in just 24 hours which ends with the hatching of a wormlike first instar larva which grows and increases its body size to produce second and third instar larva. About three days later, third instar larvae complete their growth and pupate, undergoing metamorphosis. This process last four days in which most larval tissues disintegrate and are replaced, through the proliferation and differentiation of adult precursor tissues as imaginal discs, to produce adult structures. After metamorphosis completion, imagoes emerge from the pupa case.

5.1. The heart of Drosophila melanogaster

Drosophila's heart is a simple linear tube that pumps and delivers hemolymph through the organism in an open circulatory system (Curtis et al. 1999). It is constituted of two major cell types: the inner contractile muscle cells (cardiomyocytes) are flanked on each side by pericardial cells, which do not express muscle-specific structural proteins and are implicated in hemolymph detoxification. The dorsal vessel is surrounded by an extracellular matrix composed of pericardin, a type IV collagen-like protein (Chartier et al. 2002). The adult heart forms during metamorphosis by a remodelling of the larval cardiomyocytes without cell proliferation or migration (Bodmer 2006). The dorsal vessel is divided into heart proper and aorta.

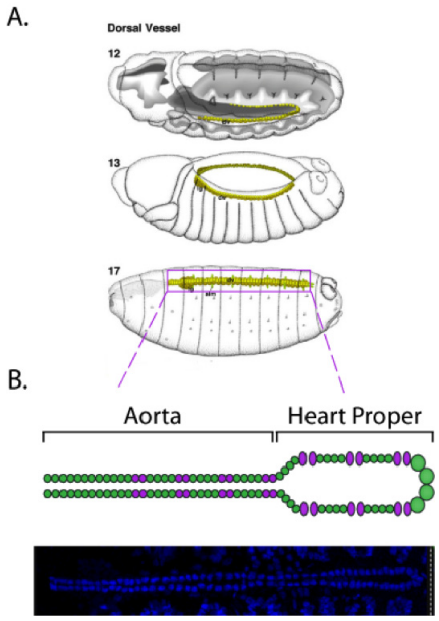

Figure 5. The embryonic Drosophila circulatory system. (A) The developing embryonic circulatory system arises from cardiac precursor cells that migrate to form the dorsal vessel at Stage 16. Stages 12, 13, and 17 are shown. Figure adapted from Fly Embryo RNAi Project . (http://flyembryo.nhlbi.nih.gov).
(B) The embryo cardiac organ is constituted by a simple tube, divided into an anterior "aorta", and a posterior "heart". Ostia cells are represented in purple; *Tin* positive cells are represented in green.
Lower Panel: Dorsal view of a stage 16 labeled with anti-Mef2 (Blue).

Cardiogenesis in flies begins with cell fate specification in the developing mesoderm where secreted ligands, including decapentaplegic (dpp), regulate embryonic heart development and trigger cell fate specification (Bodmer and Venkatesh 1998; Cripps and Olson 2002; Zaffran and Frasch 2002). Cardiac precursor cells expressing specific transcription factors including *tinman* (*tin*), the fly orthologue of *Nkx2.5*, and GATA family members migrate along the mesoderm and form the recognizable single layered dorsal vessel at stage 16 of embryonic development (Figure 5) (Bodmer 1993). It is worth mentioning that many of the temporal and tissue specific signals required for heart development are evolutionarily conserved from flies to mammals.

The newly hatched larvae possess a functional heart that pumps hemolymph from the posterior to the anterior region of the animal. During larval growth cardiomyocyte number remains constant but cells elongate dramatically, suffering changes at the ultrastructural level while the luminal space build by cardiomyocytes increases significantly (Curtis et al. 1999; Monier et al. 2005).

During metamorphosis, the heart undergoes a remodelling process to give rise to the adult heart wich remains unchanged until death (Monier et al. 2005; Zeitouni et al. 2007). It consists in a single layer of cardiomyocytes with circumferentially oriented myofibers and it is closely juxtaposed to the ventral longitudinal muscle and sets of suspensory muscles arising from the dorsal cuticle called alary muscles (Figure 6 A). The ventral longitudinal muscle is a non-cardiac muscle type. It arises from lymph cells via trans-differentiation during pupal stages of development (Shah et al. 2011).

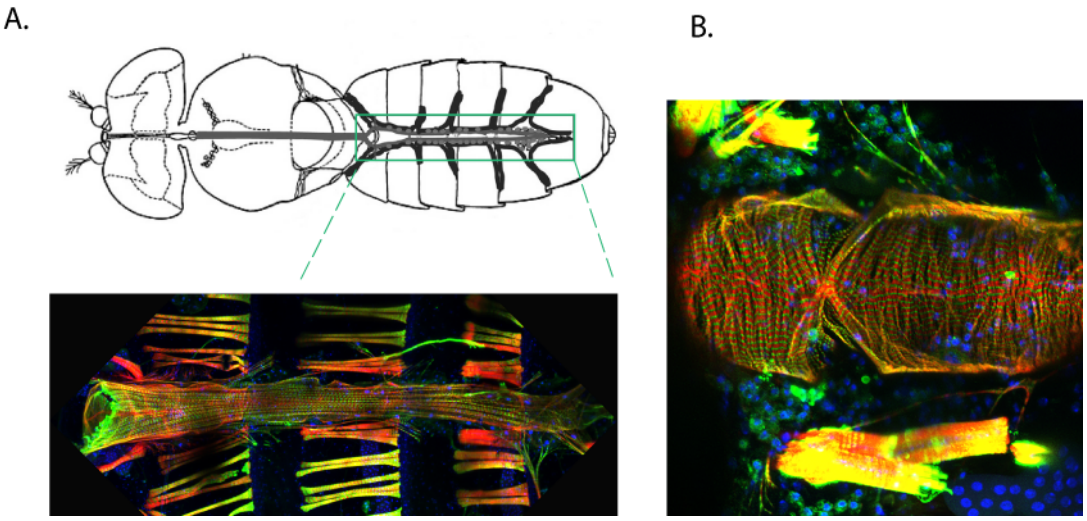

Figure 6. Adult Drosophila circulatory system. (A) The adult fly circulatory system consists of an open system with the main conical chamber, heart, located along the dorsal aspect of the A1 abdominal segment. Confocal imaging of the dorsal vessel labeled with anti-αMyosine (Green), Alexa Fluor 594-phalloidin and Dapi (blue). **(B)** Ostia cells of A3 abdominal segment labeled with anti-αMyosine (Green), Alexa Fluor 594-phalloidin and Dapi (blue).

The 104 embryonic cardiomyoblasts differentiate into three different cell types, contractile cardiomyocytes forming the heart lumen, segmentally arranged pairs of ostia cells, hemolymph inflow devices, and the intracardiac valves that divide the heart in chambers. Hemolymph enters throught four pairs of ostia cells (Figure 6B), which open during diastole and close during systole. As the heart contracts, hemolymph passes through the aorta and is dispersed into the body cavity close to the brain to re-enter the

dorsal vessel through the ostia (Shah et al. 2011).

5.2. Drosophila melanogaster as a model to study human cardiomyopathies

Drosophila melanogaster has been used to study genetic, development, and signalling for nearly a century but only over the past few decades has this tremendous resource been focus on cardiovascular research. The fruit fly is a powerful genetic model system with multitude of tools for manipulating genes and gene expression. Thus several groups have begun to examine heart function in *Drosophila* with the goal of using this system as a physiological model (Birse et al. 2010; Melkani et al. 2013; Tang et al. 2013).

Drosophila is an advantageous model for studying cardiac development and cardiac dysfunction since the genetic network controlling cardiac specification and differentiation as well as many aspects of heart function, are conserved from flies to mammals (Bodmer 1995; Bodmer and Venkatesh 1998; Cripps and Olson 2002; Harvey 1996; E. N. Olson 2006; Wolf and Rockman 2011). In addition, it is now clear that the *Drosophila* heart undergoes cardiac aging. The fly heartbeat becomes irregular with increased episodes of arrhythmias (Ocorr et al. 2007a), reminiscent of increased atrial fibrillation and heart failure in aged humans (Lakatta 2003). Most importantly, unlike in vertebrates, the fly heart is not needed for oxygen delivery and its function can therefore be significantly compromised without causing immediate death, making it an excellent model to study cardiomyopathies (Ocorr et al. 2007a).

Genetic manipulations of ion channel genes in *Drosophila*, including L-type Ca2+ channels and several types of K+ channels, perturb proper heart function, further underlining the remarkable functional parallels in basic cardiac physiology between flies and humans (Ocorr et al. 2007b; Ray and Dowse 2005; Sanguinetti and Tristani-Firouzi 2006). Furthermore, *Drosophila* constitute a useful and emerging model for the study of high fat diet induced obesity and cardiac dysfunction, one of the main causes of cardiovascular diseases in North America and Europe (Birse et al. 2010) and for the screening and identification of candidate genes involved in heart function and disease (Neely et al. 2010). Thus, *Drosophila* has become a powerful genetic model system for the study and understanding of cardiomyopathies (10.1038/ng.2610 2013) and, the recently established heart function assays make it possible to analyse and characterize adult cardiac function and functional abnormalities (Fink et al. 2009; Ocorr et al. 2007b; Ocorr et al. 2009).

Human cardiomyopathies can be categorized as dilated, hypertrophic and restrictive (Wolf 2012). Despite the simplicity of *Drosophila´s* heart, the concept of cardiomyopathies in the fly requires further definition. Dilated cardiomyopathies, the best characterized cardiomyopathies in *Drosophila*, can be defined as an "eccentric" hypertrophy in which contractile fibers are added in series and cause an enlarged chamber at end diastole and impaired systolic function. Hypertrophic cardiomyopathies can be thought of as a "concentric" hypertrophy in which contractile fibers are added in parallel, causing increased cardiomyocyte size and decreased chamber lumens. Restrictive cardiomyopathy can be thought as a process that impair heart relaxation (Wolf 2012).

6. *p53*

The tumor suppressor protein p53 is undoubtedly one of the most studied proteins to date being frequently mutated in cancer (Greenblatt et al. 1994). p53 is a transcription factor that functions in a complex signalling network to mediate cellular adaptation to stress (reviewed in (Jones and Thompson 2009). In the absence of cellular stress, p53 is present in the cell at low levels, being in an inactive state. Stimulus as genetic or metabolic stress and DNA damage lead to p53 protein activation (Harris and Levine 2005). In response to stress, p53 selectively regulates the expression of its target genes, inducing cell cycle arrest, apoptosis or senescence (Vogelstein et al. 2000).

p53 protein levels are regulated primarily by the ubiquitin ligase Mdm2, which binds to its transactivation domain and ubiquitylates the protein targeting it for degradation (Hu et al. 2007). Because p53 transcriptionally activates Mdm2, expression levels of p53 and Mdm2 are balanced through a negative feedback loop which is altered by an increase in p53 levels as result of a stress response (Hu et al. 2007). Additional Mdm2-like proteins as Mdm4, also known as MdmX, have been described to regulate p53 functions (Marine and Jochemsen 2004).

The interplay between p53, Mdm2 and Mdm4 at molecular level is complex. Mdm4 represses p53 transactivation but neither can stimulate p53 nuclear export nor its degradation, appearing to protect p53 from Mdm2-mediated suppression (Jackson and Berberich 2000). In response to cellular stress, protein-protein interactions between p53 and Mdm2 diminish, resulting in reduced p53 ubiquitination and accumulation of p53 protein (Momand et al. 2000; Shvarts et al. 1996). Once stabilized, p53 undergoes various

posttranslational modifications, including acetylation, methylation, ubiquitination and phosphorylation of specific p53 residues, that affect its activity and subcellular localization (Kruse and Gu 2008).

Although the primordial function of p53 is that of a stress-response transcription factor, recent studies indicate that it also contributes to the maintenance of intracellular homeostasis, regulating metabolism even in the absence of acute stress (Olovnikov et al. 2009; Vousden and Ryan 2009) thereby exerting prosurvival functions and regulating a number of physiological and pathological processes (Lassus et al. 1996).

6.1. Regulation of energy metabolism by p53

p53 is an emerging regulator for metabolic homeostasis, coordinating stress responses with changes in cellular metabolism. In 1956, Otto Warburg found that unlike the majority of normal cells which depend on mitochondrial oxidative phosphorylation to provide energy, most tumor cells primarily utilize glycolysis for their energy needs even under normal oxygen concentrations. This shift from mitochondrial respiration to aerobic glycolysis is known as the Warburg effect (Warburg 1956). The metabolic alterations in cancer cells determine how cells respond to variable nutrient and oxygen availability and promote cell proliferation, growth and survival (Vousden and Ryan 2009). Recent findings indicate that p53 plays an important role in metabolic shifting in cancerous cells, suggesting a new function of p53 as a cell metabolism regulator (Bensaad et al. 2006; Feng and Levine 2010; Momota et al. 2013; Vousden and Ryan 2009). In fact, p53 activation by metabolic stress is regulated by AMPK-dependent phosphorylation and influenced by mTOR (Hardie 2004), two master regulators of cellular metabolism. Thus, p53 is able to contribute to the regulation of glycolysis, oxidative phosphorylation, glutaminolysis, insulin sensitivity, nucleotide biosynthesis, mitochondrial integrity, fatty acid oxidation, antioxidant response, autophagy and mTOR signalling (Inoki et al. 2003; Maddocks and Vousden 2011)

6.1.1. The role of *p53* in regulating glycolysis and mitochondrial oxidative phosphorylation

Recent studies indicate that p53 plays a major role in suppressing glucose consumption and antagonizing the Warburg effect. It modulates glucose consumption

and glycolysis at multiple levels. p53 regulates glucose uptake by reducing, through the inhibition of NF-κB, the expression of GLUT3 glucose transporter which, together with GLUT1, is expressed in most mammalian cells (Kawauchi et al. 2008a, 2008b). It directly suppresses GLUT1 and GLUT4 expression, the later an insulin-regulated glucose transporter (Schwartzenberg-Bar-Yoseph et al. 2004). Furthermore, p53 represses the insulin receptor promoter providing another mechanism by which p53 can limit the transport of glucose into the cells (Webster et al. 1996).

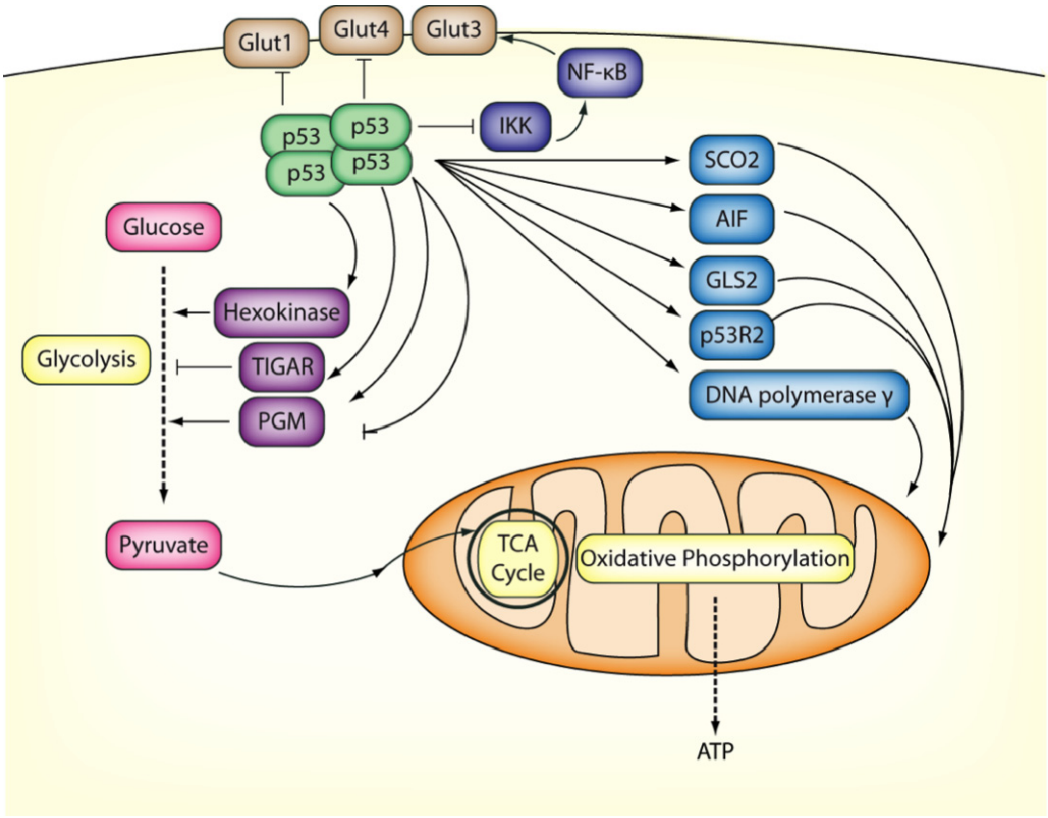

Figure 7. Regulation of energy production by p53. Several functions of p53 reduce the flux through the glycolytic pathway and increase oxidative phosphorylation, thereby opposing the Warburg effect, in which cancer cells predominantly use glycolysis for energy production. However, there are also activities of p53, such as the activation of hexokinase and phosphoglycerate mutase (PGM), which could increase glycolysis under some circumstances. GLUT, glucose transporter; IKK, IκB kinase; NF-κB, nuclear factor-κB; SCO2, synthesis of cytochrome c oxidase 2; TCA, tricarboxylic acid.

Through post-translational modifications, p53 decreases the levels of phosphoglycerate mutase (PGM), which catalyzes the conversion of 3-phosphoglycerate to 2-phosphoglycerate, promoting it´s degradation (Kondoh et al. 2005). p53 transactivates TP53-induced glycolysis and apoptosis regulator (TIGAR) (Bensaad et al. 2006), which functions as a fructose-2,6-bisphosphatase. TIGAR limits the activity of phosphofructokinase 1 (PFK1), the most important control site for the glycolytic flux,

blocking glycolysis at the fructose-6-phosphate stage and promoting the diversion of glycolytic intermediates into the pentose phosphate pathway (PPP) (Bensaad et al. 2006) (Figure 7). p53 deficient cells shows an increased PPP flux, NADPH production and glucose consumption, which can be largely reversed via the inhibition of Glucose 6-Phosphate Dehydrogenase (Jiang et al. 2011). p53 also negatively regulates the expression of pyruvate dehydrogenase kinase 2 (PDK2), which inactivates pyruvate dehydrogenase complex (PDC), a protein that converts pyruvate to acetyl-CoA, favouring the production of acetyl-CoA at the expense of lactate production (Contractor and Harris 2012).

Despite the convincing evidence that p53 can be a negative regulator of glycolyisis, there is also evidence that suggest that p53 can enhance some steps on this pathway. Hence, while p53 reduces the protein levels of PGM in embryonic fibroblast cells, in cardiac myocytes it activates the transcription of the muscle isoform of PGM (PGM-M) and hexokinase II (HK2), which catalyses the first step in glycolysis (Ruiz-Lozano et al. 1999) .

On the other hand, p53 plays a critical role in the maintenance of oxidative phosphorylation and mitochondrial integrity. It has been shown to promote OXPHOS through mechanisms that include the transcriptional activation SCO2 (Matoba et al. 2006). In addition to SCO2, p53 appears to promote mitochondrial function through different target genes, including AIF (apoptosis-inducing factor), required to maintain the integrity of mitochondrial CI (Stambolsky et al. 2006; Vahsen et al. 2004), GLS2 (glutaminase 2) (Hu et al. 2010), parkin (Zhang et al. 2011), p53R2 (p53-inducibel ribonucleotide reductase), TFAM, DNA polymerase γ (POLG) and PGC1α (Achanta et al. 2005; Bourdon et al. 2007; Kulawiec et al. 2009; Lebedeva et al. 2009; Park et al. 2009; Sahin et al. 2011) required for mitochondrial dynamics regulation and the maintenance of the mitochondrial genome.

In conclusion, given that p53 helps to shift ATP production from glycolysis to oxidative phosphorylation and since they have a homeostatic relationship, in order to regulate global changes in cellular activity the existence of a feedback mechanism between mitochondria and p53 is expected.

6.2. *p53*, mitochondria and apoptosis

Apoptosis, a type of programmed cell death, is a highly regulated process used to

remove unwanted cells. Apoptosis is required for normal development, maintenance of tissue homeostasis and regulation of certain disease states (Thompson 1995; Vaux and Korsmeyer 1999).

The proapoptotic function of p53 constitutes the best characterized facet of its oncosuppressive activities. Mitochondria is central for carrying out the intrinsic apoptosis pathway, and p53 participates in executing the intrinsic pathway through transcription dependent and independent mechanisms (Moll and Zaika 2001). p53 has been shown to induce apoptosis through the expression of a variety of pro-apoptotic genes, as Apaf-1, PUMA, Noxa, Bax, Bid, Bad and p53IAP1 (Brady and Attardi 2010; Miyashita and Reed 1995; Nakano and Vousden 2001; Robles et al. 2001) and the repression of antiapoptotic genes such as bcl-2 and survivin (Hoffman et al. 2002; Shen and Shenk 1994).

The last decade of research, has revealed the transcription independent proapoptotic activities of p53, which involves its direct action at the mitochondria (Green and Kroemer 2009; Vaseva and Moll 2009). In response to stress stimuli, a pool of cytoplasmic p53 rapidly translocates to the mitocondrial surface where it physically interacts with both anti-apoptotic (Bcl-xL, Bcl-2, Mcl-1) and proapoptotic (PUMA, Bax, Bak) members of the Bcl-2 family to inhibit or activate their respective functions, leading to mitochondrial outer membrane pore (MOMP) opening and apoptosis (Vaseva and Moll 2009).

p53 gene is a major proapoptotic factor in both *Drosophila* and vertebrates (Kanda and Miura 2004; Steller 2000). The *Drosophila* homolog of *p53*, *dp53*, has mostly a proapoptotic role, having been reported to activate the expression of Reaper and Hid proapoptotic proteins upon different insults (Brodsky et al. 2000; Fan et al. 2010; Grether et al. 1995a).

Similar to the mammalian cell death machinery, *Drosophila* possesses also a complicated cell-death regulatory system: the fly has an Apaf-1-like protein (dARK) (Kanuka et al. 1999; Rodriguez et al. 1999), seven caspases (Chen et al. 1998; Dorstyn et al. 1999; Hay and Guo 2006; Song et al. 1997) and two Bcl-2 family members (reviewed in (Igaki and Miura 2004). *Drosophila* also has unique killer proteins, Reaper, Hid and Grim (Chen et al. 1996; Grether et al. 1995b; White et al. 1994) thought to be functional orthologes of mammalian mitochondrial IAP inhibitors such as Smac/DIABLO and HtrA2/Omi.

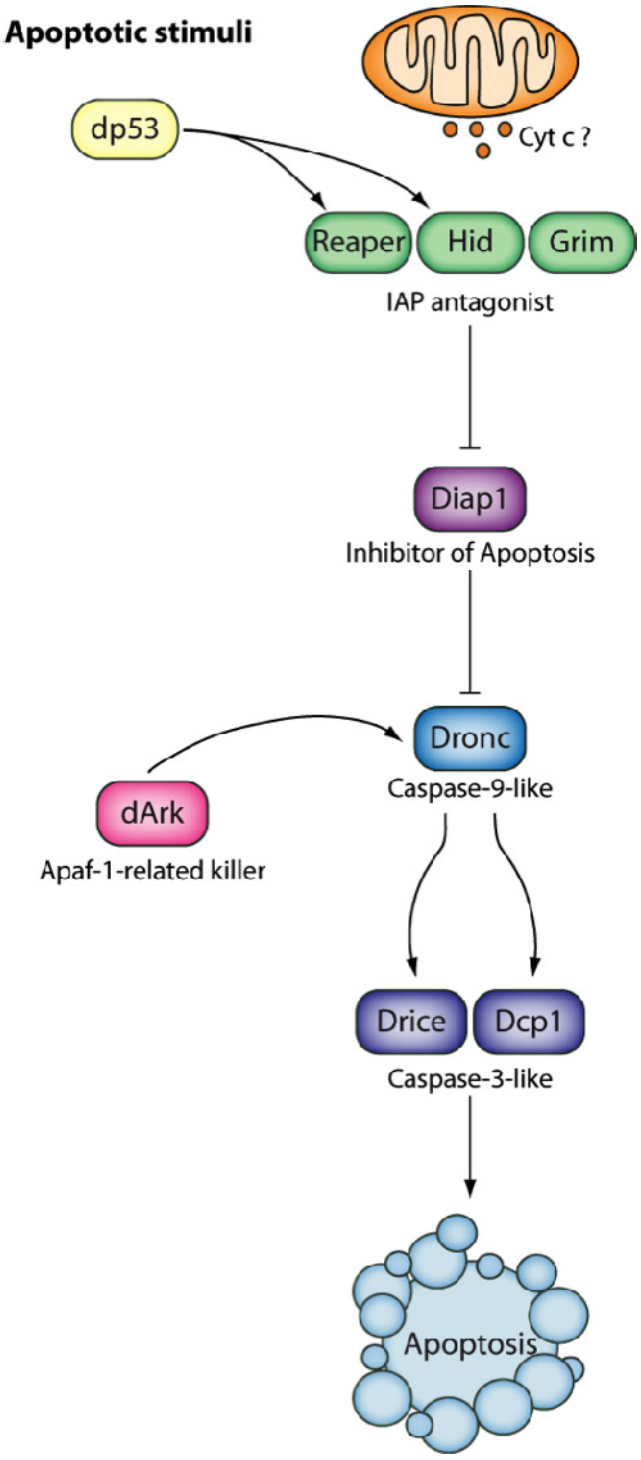

Figure 8. Schematic representation of apoptosis pathway in *Drosophila*. The adaptor protein dARK promotes activation of the apical caspase DRONC. DIAP1-binding proteins such as RPR, HID, GRIM partly promote death by disrupting 'the anti-caspase' function of DIAP1.

Caspases are the core of the cell-death machinery (Degterev et al. 2003), becoming activated in response to different death signals. As it has been mentioned above, seven *Drosophila* caspases have been identified. Like the mammalian ones, these can be divided into initiator and executioner caspases. DRONC, the fly homolog of mammalian caspases-2 and -9, has emerged as the essential apoptotic initiator caspase in *Drosophila*. DRONC interacts with dARK, and once is activated it cleaves and activates the executioner caspases DCP-1 and DRICE (Igaki et al. 2002; Kanuka et al. 1999)(reviewed in (Salvesen and Abrams 2004). This activation of DRONC might be regulated by the pro- and anti-apoptotic multidomain Bcl2 family members DEBCL and Buffy (reviewed in (Igaki and Miura 2004). Genetic studies have demonstrated that dARK and caspases act downstream of Reaper, Hid, and Grim. Thus, in cells committed to die, apoptosis is initiated by Reaper, Hid, and Grim, which bind to apoptosis inhibitor DIAP1 (Wang et al. 1999), a fly homolog of the mammalian IAP proteins, inducing its autoubiquitination and proteasome-mediated degradation, thus realising caspases from DIAP1 inhibition (Figure 8) (Steller 2008).

Although the role of mitochondria in *Drosophila* apoptosis its still unclear and despite the striking differences, there are some clear evidence suggesting that mitochondria play an important role in cell death in flies as it does in mammals. In fact, it is known that Rpr, Hid and Grim localization in the mitochondria is essential to promote cell death (Claveria et al. 2002; Haining et al. 1999; M. R. Olson et al. 2003), and it has been shown that under apoptotic stimulus, fly mitochondria undergoes Rpr, Hid and Drp1-dependent morphological changes and disruption during cell death (Abdelwahid et al. 2007). The participation of Drp1, a mitochondrial fission protein, in cell death is conserved in worms and mammals (Frank et al. 2001; Jagasia et al. 2005).

AIMS

COX deficiency due to mutations in COX assembly factors is one of the most frequent causes of MRC defects in humans. Usually, it has a very early age of onset and fatal outcome, displaying different clinical presentations, including encephalopathies such as Leigh Syndrome, fatal cardiomyopathy, hepatic failure and leukodystrophy.

Pathogenic mutations in human Sco1 and Sco2 have been reported to cause hypertrophic cardiomyopathy among other clinical symptoms. Although the role of Sco proteins as copper metallochaperones is largely documented, the molecular mechanism underlying the cardiac dysfunction caused by mutations in human Sco1 and Sco2, have yet to be elucidated.

Thus, the generation of animal models is of vital importance. Although unfortunately there are no Sco1 KO mice available, a $Sco2^{KIKO}$ human disease mice model has been recently developed. Despite $Sco2^{KIKO}$ mice model present most features of the human disease they showed no evidence of cardiomyopathy.

Therefore, the specific aims of the present study were:

1. Generation of a *Drosophila* cardiomyopathy model to study cardiomyopathies with a mitochondrial origin.

2. Investigate the genetic and molecular mechanisms that underlie ScoX induced cardiomyopathy.

MATERIALS AND METHODS

1. Materials

1.1 Reagents, solutions and buffers

All the reagents used in the different experiments performed in this thesis are described in methods or as described in the references.

1.2. Drosophila melanogaster

1.2.1. Standard condition for *Drosophila* growth

All fly stocks were raised on standard *Drosophila* medium; yeast 10%, sucrose 7.5%, wheat flour 3.5%, propionic acid and agar 1.25%.

Fly culture and crosses throughout this thesis were performed at 25ºC and 60% relative humidity.

1.2.2. Fly Stocks

All fly stocks used in this thesis are listed in the below, tables 1 to 3. For RNAi silencing, Scox-RNAi or Surf1-RNAi males or virgin females were crossed to *TinCΔ4-Gal4* flies and incubated at 25ºC throughout development. Female F-1 progeny were

collected for posterior analysis and aged at 25ºC. *w*^{1118} flies crossed to *TinCΔ4-Gal4* or each UAS-RNAi line were used as control flies.

1.2.2.1 Balancers and control stocks

Name	Origin	Observations
yellow white	R. Garesse	Control stock
w^{1118}	R. Garesse	Parental transgenic stock
Double Balancers	R. Garesse	Used for the mapping of P elements in the transgenic lines generated

Table 1.

1.2.2.2. Gal4 stock

Name	Origen	Observations
daugtherless-GAL4	Bloomington Stock Centre nº 8641	Ubiquitous
TinCΔ4-Gal4	Manfred Frasch	Cardiac-tissue specific

Table 2.

1.2.2.3 UAS stocks

Nombre	Origen	Observaciones
UAS - ScoxRNAi	Vienna Drosophila RNAi Center (VDRC)	Scox RNAi of *Drosophila melanogaster*
UAS – Surf1RNAi	Vienna Drosophila RNAi Center (VDRC)	Surf1 RNAi of *Drosophila melanogaster*
UAS – p53	Rolf Bodmer	Overexpression of p53

UAS – $p53^{DN}$	Rolf Bodmer	Overexpression of a DN form of p53
UAS- p53 mut	Bloomington Drosophila Stock Center	Deletion of dp53 gen
UAS- GFP Stringer	Rolf Bodmer	Green fluorescent protein
UAS-p35	Manuel Calleja	Inhibitor of apoptosis
UAS-Diap1	Manuel Calleja	Inhibitor of apoptosis
UAS-Rpr	Manuel Calleja	Pro-apoptotic gene

Table 3.

1.3. Oligonucleotides

The oligonucleotides used in the development of this thesis were synthesized by Sigma-Aldrich. Oligonucleotides names and 5´→3´ sequences are listed in Table 4.

Nombre	**Secuencia**
Rpl10 Fw	AAGAAGGTGCTCTGCCTGTC
Rpl10Rv	CGCACATTCTGCCAGTTCT
Scox Fw	CTCCCGCAGATTCCACTAAA
Scox Rw	GCTCTTCACGTACAGCATGA
IMPL3 Fw	GTGTGCCTCATCGATGTCTG
IMPL3 Rw	GTGCTGGCCGTGATCTG
GPI Fw	GGCCTTCACTCCCACTTTTGT
GPI Rw	CCACGGACTCAGGCTCCT
Pdk Fw	ATCTCATTAGGAATCGGCACAA
Pdk Rw	CTGAATGGAACTCTCTGTAGGC

Pfk Fw	CGCCTAGCTGTGATGCATATT
Pfk Rw	GACACCGTCGTTGATTCCATAA
Rpr Fw	GAGTCACAGTGGAGATTCCT
Rpr Rw	AATCCTCATTGCGATGC
Hid Fw	ACGGCCATCCGAATCCGAAC
Hid Rw	TGCTGCTGCCGGAAGAAGAAGTT
Grim Fw	CATCAGCAACAGCGCCCA
Grim Rw	GCTGGCTCGAACTGTAGCTG
UAS Fw	CACACCACAGAAGTAAGGTTCCTTCAC
UAS Rw	GAACACGTCGCTAAGCGAAAGCTAAG
Gal4 Fw	TCCTCGAGAAGACCTTG
Gal4 Rw	GGTCCGTTTTCAGGAAG

Table 4.

1.6. Dyes

- Dapi (Molecular Probes)
-Phalloidin-TRITC (Molecular Probes)
-Phalloiding-488 (Molecular Probes)

2. Methods

2.1. GAL4/UAS system

In this thesis, we have used the GAL4/UAS system for targeted gene expression in *Drosophila melanogaster* (A. H. Brand and Perrimon 1993a). This system allows the ectopic expression of a gene of interest (gene X) in a specific tissue or cell type (Figure 9). The expression of the gene of interest is controlled by the UAS element, consisting of GAL4-binding sites. GAL4 is a yeast transcriptional activator with no known binding sies

in the *Drosophila* genome; since the transcription of the gene of interest requires GAL4, its absence maintains the transgene inactive. Transgene expression is activated by crossing UAS flies to flies expressing GAL4 in a certain location, also known as "drivers". The resulting progeny will express the transgene in a specific cell or tissue type, depending on the "driver".

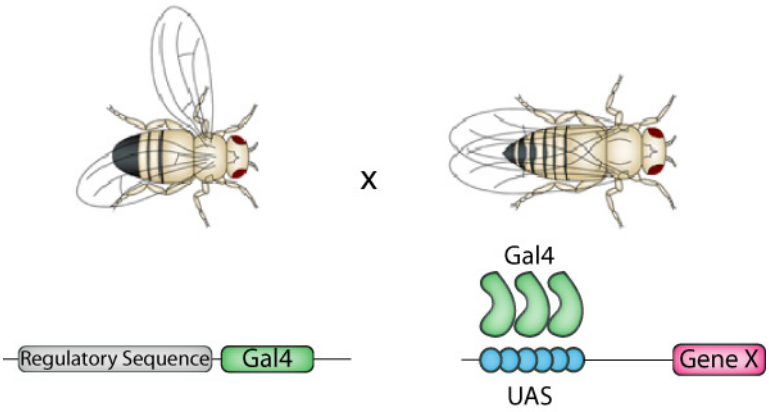

Figure 9. Squematic representation of the GAL4/UAS system.

2.2. DNA ISOLATION

2.2.1. *Drosophila melanogaster* DNA isolation

DNA isolation from *Drosophila* adults was performed as described (Gloor et al. 1993). Briefly, 1-2 flies were disrupted in 50 µl of Squashing buffer, 10 mM Tris-Cl pH 8.2, 1 mM EDTA, 25 mM NaCl supplemented with 1 µl Proteinase K 10 mg/ml. Sample was incubated at room temperature for 20-30 minutes. Then, Proteinase K was heat inactivated at 95º C for 1-2 minutes. 2 µl of DNA, were used for the PCR amplification.

2.3. *Drosophila* cardiac analysis

1 or 2 week old females were anaesthetized with Fly Nap (Carolina Biological Supply) for 2-5 min and dissected at room temperature (18-22ºC) as previously described (Fink et al. 2009; Ocorr et al. 2009). In brief, head, ventral thorax and ventral abdominal cuticule was removed exposing the abdomen. Afterwards, all internal organs and fat were removed in order to expose the heart. Dissections were performed in oxygenated artificial hemolymph (Ocorr et al. 2009) .Semi-intact preparations were allowed to

equilibrate with oxygenation for 20 min prior to filming. Movies of beating hearts were recorded for 30 seconds with a high-speed Hamamatsu EM-CCD digital camara at rates of 100-200 frames/sec in conjunction with 10x immersion lens. Movies were analyzed with Semi-automatic Optical Heartbeat Analysis software to quantify heart periods, systolic and diastolic intervals, systolic and diastolic diameters, cardiac rhythmicity, frantional shortening and to produce M-mode records (Fink et al. 2009).

2.4. Inmunostaining of the Adult *Drosophila* heart

Fluorescence imaging of *Drosophila* heart tubes were performed as described (Alayari et al. 2009). Briefly, semi-intact *Drosophila* hearts were placed in artificial hemolymph containing 10mM EGTA for 5-10 min to inhibit cardiac tube contractions. Hearts were then fixed in situ for 20 min with 4% paraformaldehyde in PBS at room temperature with gentle shaking. Then, hearts were washed three times in PBST (PBS - 0.1% Triton X-100). After washing, hearts were incubated with Alexa Fluor-594 Phalloidin in PBST (1:200) for 2h. Hearts were washed three times for 10 min in PBST, rinsed in 100 µl of PBS for 10 min and mounted on Vectashield (Vector Laboratories). Images were acquired using a Zeiss LSM 710 fluorescent microscope.

2.5. TUNEL Staining of adult hearts

TUNEL staining was essentially performed according to the instructions from the manufacturer (In Situ Cell Death Detection Kit, TMR Red, Roche, Germany). Briefly, semi-intact hearts were prepared as described above. Then, hearts were incubated with PBST plus Na-Citrate 100mM at 65ºC for 30 min. Then the hearts were washed trice 10 minutes in PBST, incubated in TUNEL-Dilution buffer two times for 5 min at room temperature and afterwards in Labeling solution for 30 mins at 37ºC. Then, hearts were incubated in Enzyme solution at 37ºC for 2h and washed three times, 10 min in PBST, and mounted on Vectashield plus DAPI (Vector Laboratories). For positive control, hearts were pretreated with DNAse I to introduce nonspecific strand breaks.

2.6. TUNEL Staining of Mouse esqueletal muscle and liver

Muscle and liver tissues were cryo-sectioned (8µm thick) and tissue sections were fixed for 20 min at room temperature in 4% paraformaldehyde-PBS and stained for TUNEL as described above. For positive control, tissues were pretreated with DNAse I to introduce nonspecific strand breaks.

2.7. DHE Stainning

Dihydroethidium (DHE) was employed for the detection of oxidative stress. Briefly semi-intact hearts were prepared as described above and stained with 3mM DHE (Molecular Probes, Carlsbad, CA) in PBS for 1h, followed by three washes in PBS. Hearts were relaxed with 10mM EGTA and fixed in 7% paraformaldehyde-PBS for 4 min at room temperature. Hearts were mounted in Vectashield . Fluorescence imaging was carried out using a Zeiss LSM 710 fluorescent microscope.

2.8. Respiratory Complexes enzyme activity measurement

Mitochondrion-enriched homogenates were prepared from 30 third instard larvae ground in SETH buffer (250 mM sucrose, 2mM EDTA, 100 units/liter heparin, 10 mM Tris-HCL, pH 7.4), in a glass-glass homogenizer (5-8 strokes on ice). The homgenate was centrifugated at 600g for 5 min at 4ºC. The supernatant was collected and centrifugated a second time at 8000g for 15 min at 4ºC. The pellet containing the crude mitochondria was resuspended in SETH buffer. Protein concentration was determined by the Bradford method using bovine serum albumin as a standard using DC Protein Assay Kit (Bio-Rad). Respiratory chain complexes Inad IV and mitochondrial mass marker Citrate Syntase activities were measured spectrophotometricaly as described previously (Perez-Carreras et al. 2003). Activities are expressed in nanomoles of substrate catalyzed/minute, milligram of protein.

2.9. Inmunohistochemical Staining of Adul hearts

Briefly, semi-intact hearts were prepared as described above and stained for SDH and COX activities (Sciacco and Bonilla 1996). For SDH staining, hearts were incubated in

SDH staining solution (0.01M PBS, 5mM Na-EDTA, 1mM Potassium cyanide, 0.2mM Phenazine Methasulfate, 50mM Succinic acid, 1.5mM Nitro Blue Tetrazolium, pH 7.0) at 37ºC for 20 min. For control hearts, SDH staining solution was supplemented with 10mM sodium malonate. For COX activity, hearts were incubated at 37ºC in the dark for 3h with COX staining solution (0.1M PBS, 5mg 3,3´-diaminobenzidine (DAB), 2mg Cytochrome c, 0.4g Sucrose, pH 7.4). For control hearts, COX staining solution was supplemented with 10 mM potassium cyanide. Afterwards, hearts were washed three times for 10 min in PBS and fixed with 4%paraformaldehyde-PBS at room temperature for 20 min. Optical imaging was carried out using a Nikon 90i.

2.10. mRNA extraction and Q-RT-PCR of *Drosophila* cardiac associated transcripts

Total RNA was extracted from 8-10 hearts per genotype dissected from female flies. RNA extractions, Reverse Transcription (RT) and Quantitative RT-PCR were performed with the Fast SYBR Green Cells-to-CT™ KIT (Ambion, Applied Biosystems) in a 7900 Fast Real Time PCR System (Applied Biosystems) according to manufacturer´s protocol.

2.11. Statistical Analyses

Data are presented as mean values ± SEM. Q-PCRs were analyzed by a 2-tailed, unpaired Student´s *t*-test between two groups and ANOVA for multiple comparisons. For heart function, data sets were tested for normal (Gaussian) distributions using the D´Agostino and Pearson ómnibus normality test. For data sets that passed this test, we used a regular 1-way ANOVA followed by a Tukey multiple comparisons post hoc test of significance. In all cases, $P<0.05$ were taken as significant. All statistical analyses were performed using Prism Statistical Software (Graph Pad, Inc, versión 6).

RESULTS

1. ScoX knockdown in Drosophila melanogaster

Mitochondrial diseases are characterized for having a whole body impact, therefore it is very difficult to distinguish tissue specific phenotypes from systemic ones. In particular, pathogenic mutations in the human *SCO* genes, and *Sco2* in particular, cause fatal infantile mitochondrial diseases associated with COX deficiency. Mutations in the human *Sco2* have been found to cause fatal cardio-encephalomyopathy (Jaksch et al. 2000; Knuf et al. 2007; Papadopoulou et al. 1999). Patients with mutations in *Sco1* display fatal infantile encephalopathy and lactic acidosis (Leary et al. 2013) and in some cases, early onset hypertrophic cardiomyopathy, hypotoina and hepatopathy (Stiburek et al. 2009).

In *Drosophila* there is a single *Sco1* and *2* ortholog, *ScoX*, which has been recently identified and characterized (Porcelli et al. 2010). *ScoX* null mutant flies are homozygous lethal at larval stages, while weaker mutants are associated with motor dysfunction and female sterility. They also present a strong disruption of Complex IV assembly and a concomitant reduction of enzyme activity (Porcelli et al. 2010). To analyze the tissue specific role played by Sco proteins in the human cardiomyopathy, we have generated a *Drosophila* heart specific Gal4 driven RNAi model to knock down ScoX protein levels specifically in cardiomyocytes (A. H. Brand and Perrimon 1993b).

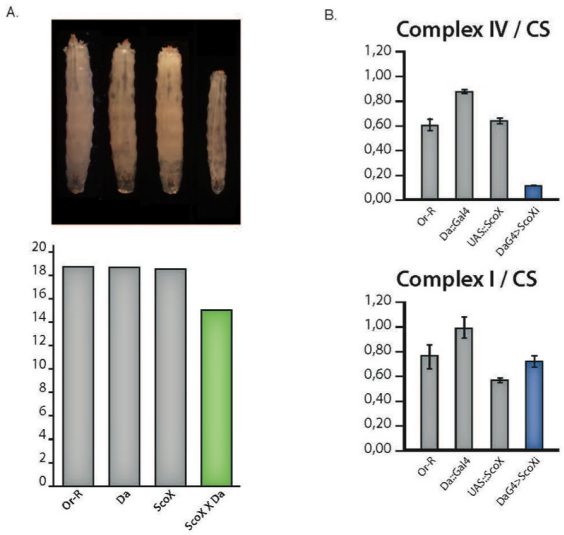

Figure 10. Ubiquitous *ScoX* knock down phenocopies mutant phenotype.
(A) Representative examples of 3rd instard larval sizes (upper panel) and mesurement of their length (lower panel) Values are means ± S.E. n= 12-15. Error bars indicate SEM.
(B) Complex IV and I enzymatic activities in mitochondria purified from 3rd instard larvae normalized for Citrate Sintase activity as an indicator of mitochondrial mass. n=2.

In order to validate our *ScoX* silencing approach, we decided to test if the ubiquitous knockdown of *ScoX* would phenocopy the described phenotypes for *ScoX* homozygous mutants. Ubiquitous *ScoX* interference driven by Daughterless-Gal4 driver is larval lethal. Animals develop until third instard larva, but none of them reach pupal stages. Third instard larvae are much smaller and thinner than controls, displaying a *Spargel* phenotype, which has been described in other mutations affecting mitochondrial proteins (Peralta et al. 2012; Tiefenbock et al. 2010) (Figure 10 A).

Moreover, in agreement with *ScoX* mutant phenotype, *ScoX* silencing results in a complex IV specific impairment of mitochondrial function. We measured Complex I and IV activity in controls and *ScoX* knockdown third instar larvae. *Da-Gal4/+>SCOXi/+* larvae showed a dramatic reduction in COX activity whereas Complex I activity remained unaffected (Figure 10 B), demonstrating that ubiquitous interference of *ScoX* expression phenocopies its loss of function and ruling out the possibility that the observed phenotypes could be due to off target effects.

2. Cardiac specific *ScoX* knockdown cause mitochondrial impairment.

In order to develop a fly model to study the role of the loss of Sco function in the development of cardiomyopathy, we followed a cardiac specific *ScoX* RNAi silencing approach using *TinCΔ4-Gal4* as the driver (Lo and Frasch 2001). First, we sought to

determine if heart specific *ScoX* knockdown compromises *Drosophila* lifespan. *TinCΔ4-Gal4>ScoXi* flies show shortened lifespan as compared to controls (Figure 11). Since *ScoX* interfered flies start to die after 2 weeks, all experiments were carried out in 1 or 2 week old flies.

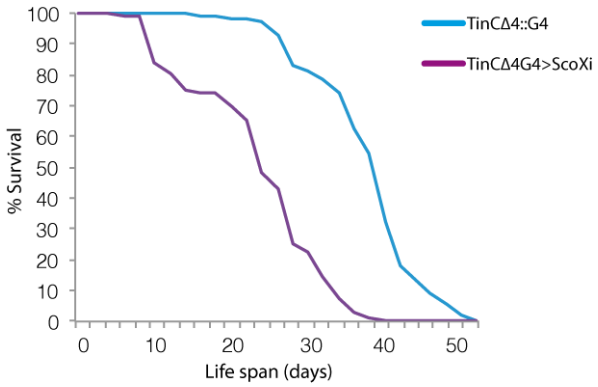

Figure 11. Survival curve. Cardiac specific *ScoX* knockdown affects the life span of the flies. Graph plots % survival (n=200 for each genotype).

2.1. Mitochondrial complexes activity staining in *ScoX* knockdown hearts

Next, we measured *ScoX* interference strength by assessing mRNA levels in fly hearts by qRT-PCR and, given the small size of this organ in *Drosophila,* performing histochemical staining of heart complex IV activity. Hearts from one week old *TinCΔ4-Gal4>ScoXi* flies showed a 50% reduction in *ScoX* mRNA levels as compared to controls, while complex IV activity histochemical staining of hearts from 1-week old control and knockdown flies demonstrated a clear deficiency in COX activity in the later (Figure 12 A and B).

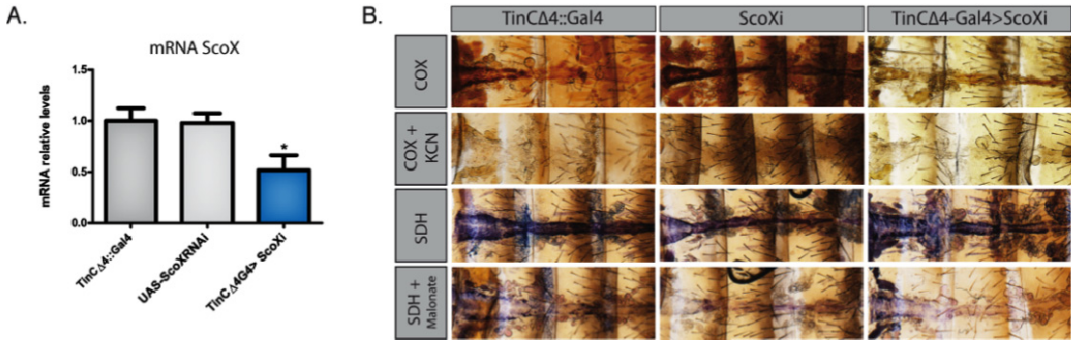

Figure 12. Cardiac specific *ScoX* knockdown cause a mitochondrial impairment.
(A) *ScoX* mRNA expression levels in *TinCΔ4::G4*, *UAS-ScoXi* and *TinCΔ4-G4>ScoXi* hearts normalized to RPL0 and relative to TinCΔ4::G4. Values are means ± S.E. n= 6-10. *p < 0.05.
(B) Enzyme histochemical staining for COX and SDH activities from *TinCΔ4::G4*, *UAS-ScoXi* and *TinCΔ4>ScoXi* hearts in absence or presence of their respective inhibitors KCN (COX) and malonate (CII).

The observed decrease in COX activity was not due to a general respiratory chain mal function since we observed no differences in succinate dehydrogenase (SDH) (Figure 12 B), or complex I (data not shown) activity staining between controls and *ScoX* interfered hearts. These results indicate that a decrease of just 50% of *ScoX* mRNA levels is enough to compromise COX activity in the *Drosophila* heart, and that this defect is specific for Complex IV.

2.2. Characterization of metabolic state in Scox knockdown cardiomyocytes

It is well described that mitochondrial dysfunction leads to an alteration in energy metabolism in many forms of heart disease (Masoud et al. 2013; Nascimben et al. 2004). We therefore hypothesized that COX deficiency displayed by *TinCΔ4-Gal4>ScoXi* hearts would alter oxidative phosphorylation resulting in a compensatory up-regulation of glycolysis and partial blockage of the Krebs cycle. To address this supposition we measured by qRT-PCR the mRNA relative levels of key enzymes involved in different steps of these process. As show in figure 13, mRNA expression levels of *gpi*, *pfk* and *pgk1* were upregulated in *TinCΔ4-Gal4>ScoXi* hearts (Figure 13).

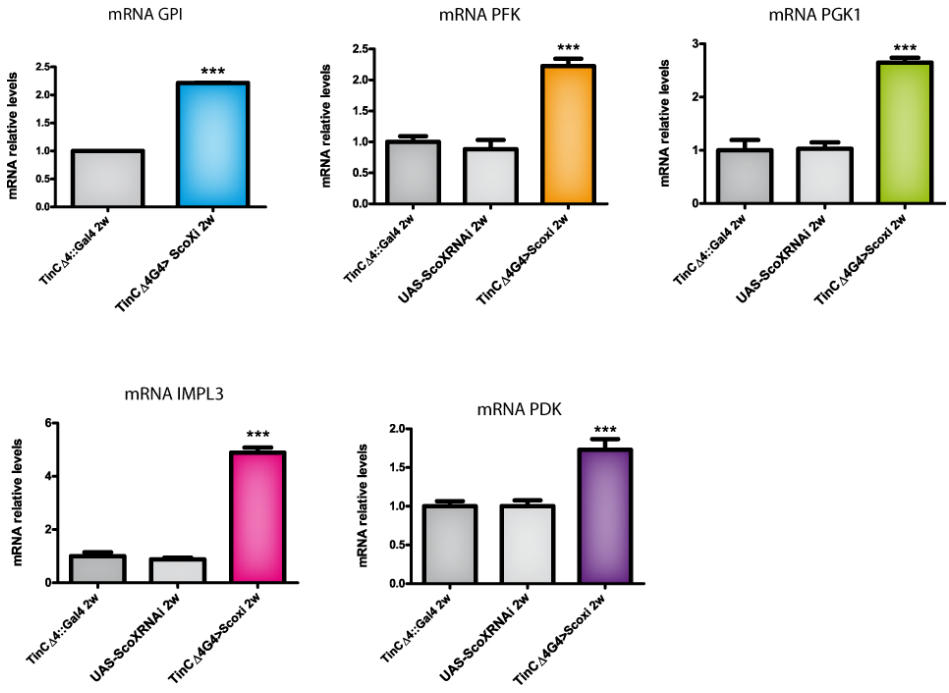

Figure 13. Cardiac specific *ScoX* knockdown cause a metabolic switch. mRNA expression levels of *PDK, GPI, PFK, PGK1* and *IMPL3* mRNA in hearts from 2-week old *TinCΔ4::G4, UAS-ScoXi* and *TinCΔ4G4>ScoXi* flies normalized to *RPL0* and relative to *TinCΔ4::G4*. Values are means ± S.E. n= 6-10. ***p < 0.001.

Furthermore, in these hearts we observed an increased expression of *impl3*, the *Drosophila* ortholog of human lactate dehydrogenase (LDH) (10.1038/ng.2610), and the mitochondrial matrix enzyme *pdk* (Figure 13). LDH enzyme converts pyruvate to lactate, the final product of the non-respiratory consumption of glucose while PDK is a key regulator of glucose oxidation. It phosphorylates and inhibits pyruvate dehydrogenase (PDH), therefore blocking the kreb´s cycle (Piao et al. 2010; Sugden et al. 2000).

Our data strongly suggest that *TinCΔ4-Gal4>ScoXi* cardiomyocytes undergo a metabolic switch from glucose oxidation to glycolysis. Since the pyruvate cannot be used by mitochondrial PDH (inhibited by PDK high levels), the concomitant increase in *impl3* mRNA relative levels led us to hypothesize that, in order to maintain the glycolytic flux, pyruvate is mostly converted to lactate resulting in lactic acidosis. Our data suggest that heart specific interference of *ScoX* results in a mitochondrial dysfunction due to the alteration of oxidative phosphorylation and promotes upregulation of glycolysis.

3. *ScoX* RNAi knockdown causes dilated cardiomyopathy in *Drosophila melanogaster*

3.1. Analysis of heart structure

To explore the impact of the mitochondrial dysfunction caused by *ScoX* silencing in the fly heart, we analyzed the structure of controls and *TinCΔ4-Gal4>ScoXi* hearts from 1 and 2 week old flies by confocal microscopy. Although overall heart structure seemed to be fairly normal, *ScoX* RNAi animals exhibited myofibrillar disarray and narrowing of the heart tube from abdominal segment 4. This degeneration of the heart tube became more noticeable with age, with 2 week old flies having a much stronger phenotype (Figure 14 A). Intriguingly, *TinCΔ4-Gal4>ScoXi* heart tubes had an apparent increase in conical chamber diameter as compared to controls. We measured the diameter of the conical chamber from 1-week old *TinCΔ4-Gal4>ScoXi* and controls hearts. As shown in figure 14 B, knocked down hearts had increased conical chamber diameter, suggesting that in *Drosophila* heart specific *ScoX* interference causes dilated cardiomyopathy.

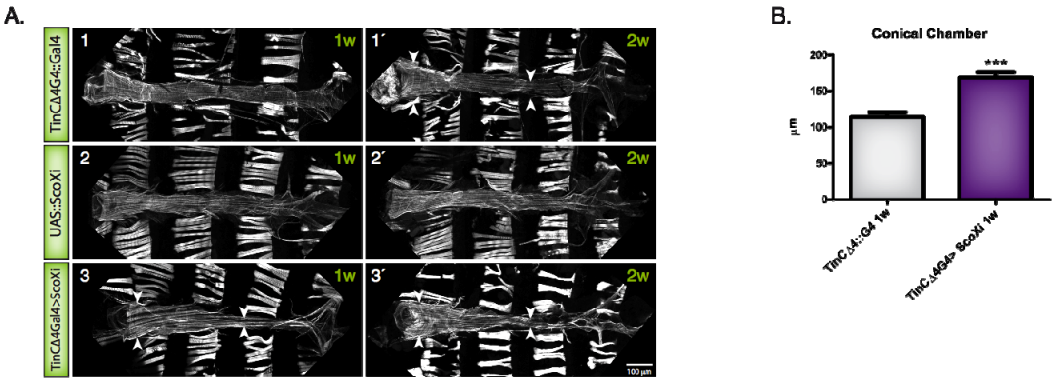

Figure 14. Cardiac-specific *ScoX* knockdown causes dilated cardiomyopathy in *Drosophila melanogaster*.
(A) Fluorescent-phalloidin staining from 1 and 2-week old adult *TinCΔ4::G4* (1-1'), *UAS-ScoXi* (2-2') and *TinCΔ4G4>ScoXi* (3-3') hearts. Arrowheads indicate the conical chamber and A4 segment.
(B) Conical chamber diameter measurement from 1-week old *TinCΔ4::G4* and *TinCΔ4G4>ScoXi* adult hearts. Values are means ± S.E. n=10. ***p < 0.0001.

3.2. *Scox* RNAi knockdown compromises heart function

We then characterized the cardiac physiology in order to investigate how *ScoX* silencing affects heart function. We used high speed optical recording of semi-intact adult preparations of beating hearts and posterior semiautomated analysis software (Fink et al. 2009; Ocorr et al. 2007b). M-mode traces obtained from high-speed movies illustrates heart wall movements over time (Figure 15 A). Although *TinCΔ4-Gal4>ScoXi* and control hearts showed regular rhythmic contractions, M-Modes from *TinCΔ4-Gal4>ScoXi* from 1 and 2-week old flies exhibited a significant prolonged heart beat length (heart period [HP]) (Figure 15 B) and a reduced heart rate compared to control hearts (Figure 15 C). The observed increase in the heart period is due to an increased diastolic interval (DI) (Figure 15 D). We quantified the number of flies displaying DIs longer than one second. 20 % of the 2-week old *TinCΔ4-Gal4>ScoXi* flies showed long DIs compared with controls (Figure 15 E). Additionally, hearts from these flies displayed a markedly constricted diastolic diameter, whereas there was little effect on the systolic diameter, which results in a diminished fractional shortening, an estimation of the contractility of the heart tube (Figure 15 F-H). We found that *ScoX* RNAi hearts exhibited a degenerative phenotype in all the cardiac parameters measured. Furthermore, the observed phenotype was not only age but also dose dependent since animals harbouring just one copy of both driver and UAS::*ScoXRNAi* develop milder phenotypes (Figure 15 A, compare *TinCΔ4-Gal4>ScoXi* to *TinCΔ4-Gal4/+>ScoXi/+*). Thus, cardiac-

specific knockdown of *ScoX* results in severe cardiac dysfunction and dilated cardiomyopathy.

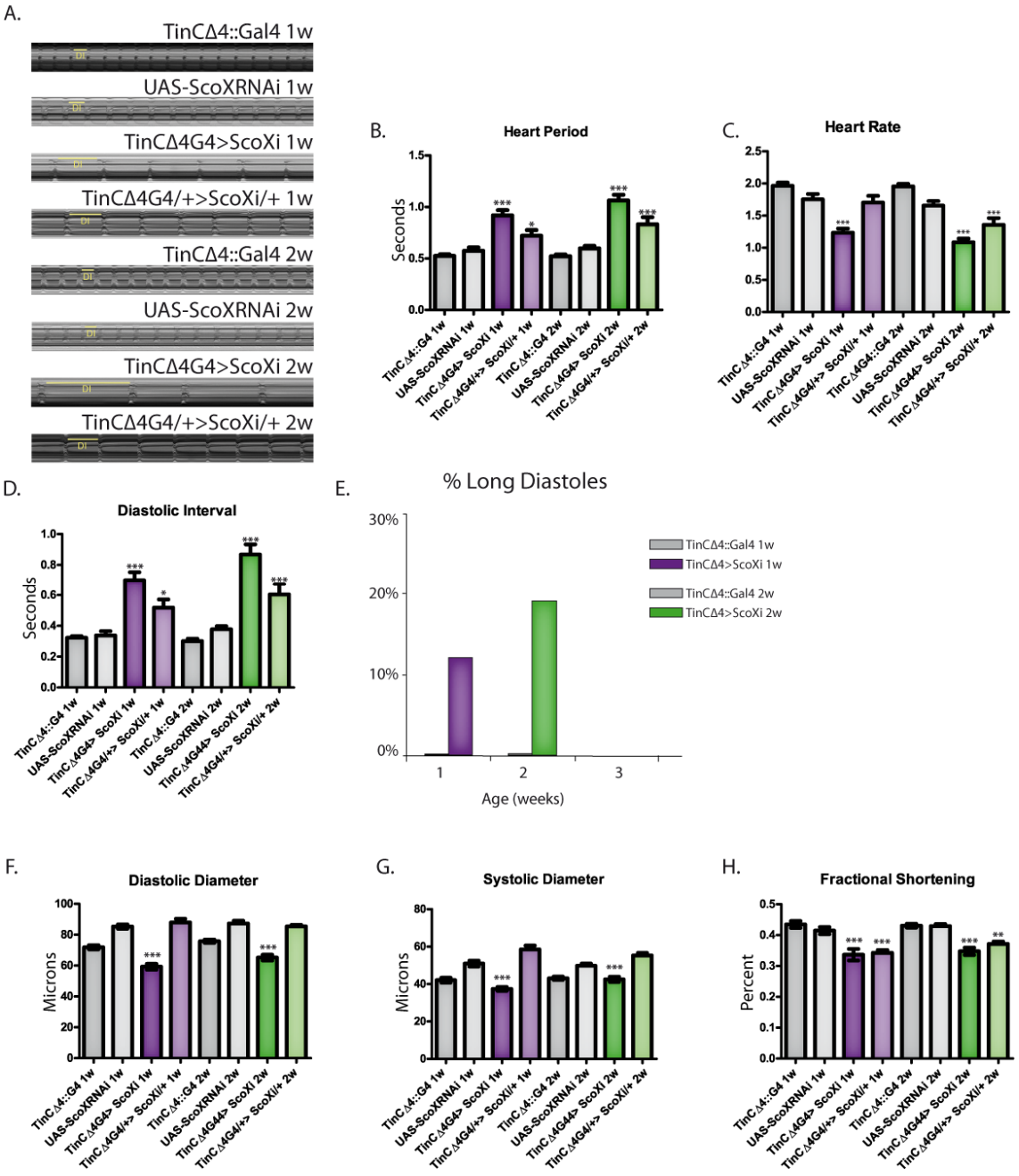

Figure 15. Cardiac-specific *ScoX* knockdown causes heart dysfunction in *Drosophila melanogaster*.

(A) Representative M-Mode traces (10s) from high-speed movies of 1 and 2 weeks old semi-intact flies.

(B-H) Physiological parameter measurement in hearts from 1 and 2 weeks old semi-intact flies. (B) Heart Period, (C) Heart Rate, (D) Diastolic Interval, (E) % of Long diastoles, (F) Diastolic Diameter, (G) Systolic Diameter, (H) Fractional Shortening. Differences are relative to *TinCΔ4::Gal4*. Values are means ± S.E. n= 20-40 flies. *=p<0.05, **=p<0.01 and ***=p<0.001).

3.3. *ScoX* knockdown affects heart structure.

The altered heart morphology and the reduced contractility made us think that cardiomyocyte myofibril structure might be altered in *ScoX* knockdown hearts. We analyzed fiber organization in cardiomyocytes from 3rd and 4th abdominal segments (A3 and A4) in 1 and 2-week old flies by phalloidin staining and fluorescent microscopy. Hearts from controls contained tight and well-aligned circumferential myofibrils within the cardiomyocytes (Figure 16 A and B 1-2). However, cardiomyocyte myofibers from *TinCΔ4-Gal4>ScoXi* animals were loosely packed and poorly organized, being fully disorganized in those hearts displaying the "stronger" phenotypes. As expected, disorganization is stronger in the posterior half (compare A3 and A4 segments, Figure 16 B). These phenotypes are degenerative and 2-week old flies display extremely severe myofibrillar disarray with the heart tube often looking completely collapsed (Figure 16 A 3-4´; Figure 16 B 3-4´).

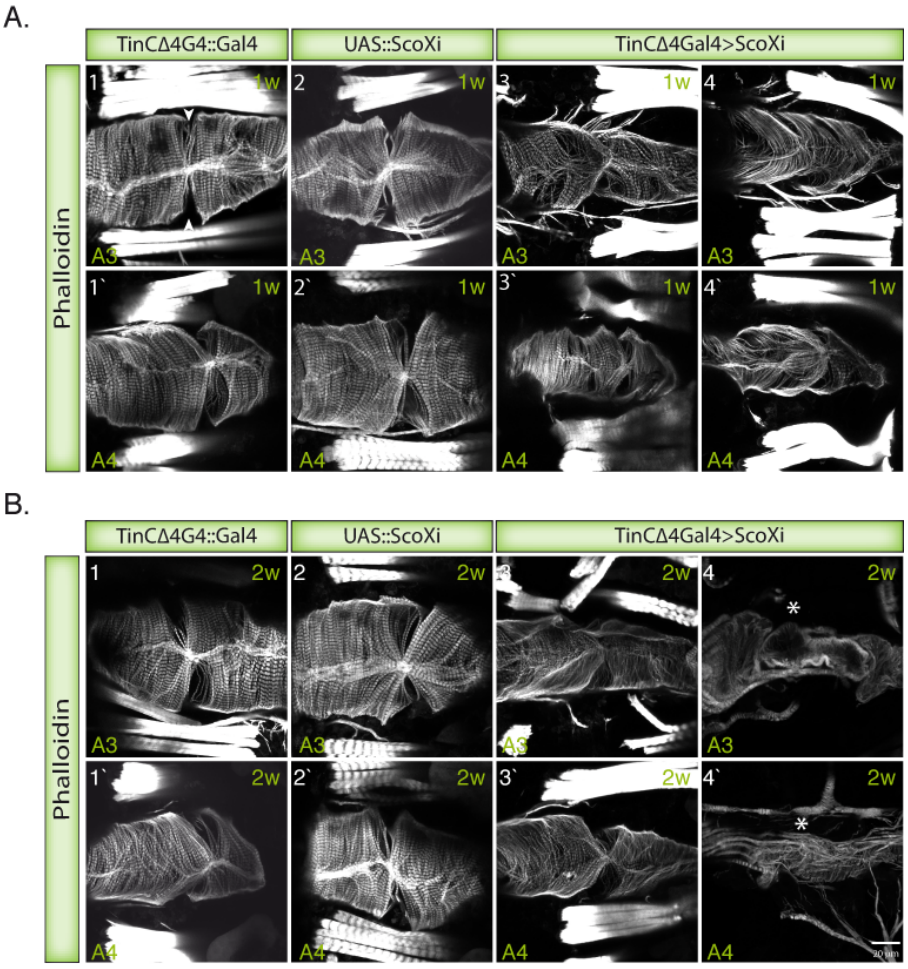

Figure 16. Cardiac-specific knockdown of *ScoX* affects heart tube structure. Fluorescent-phalloidin staining of 3rd and 4th abdominal segment (A3 and A4) of the dorsal vessel from 1 **(A)** and 2 **(B)** week old adult *TinCΔ4::G4* (1-1'), *UAS-ScoXi* (2-2') and

TinCΔ4G4>ScoXi (3-4') hearts. Arrows- Missing myofibrils. *- Circular myocardial myofibrils completely disrupted.

Since one case of fetal wastage harboring SCO2 mutations has been reported in humans (Tay et al. 2004), we wondered if the structural defects observed in *TinCΔ4-Gal4>ScoXi* heart tube were the consequence of a developmental defect or if the degenerative process was a result of the described mitochondrial dysfunction. To answer this question, we next examined heart tubes from pupa of controls and *TinCΔ4-Gal4>ScoXi* flies. Fluorescent imaging revealed that pupa from *TinCΔ4-Gal4>ScoXi* flies exhibited normal heart structure compared to controls (Figure 17), ruling out the possibility that the age related myofibril disorganization could be the consequence of a developmental defect. Taking all these results together, and consistent with the data obtained from live beating hearts, these data suggest that *ScoX* down-regulation compromises heart function and structure in an age related manner and that this is not due to developmental defects.

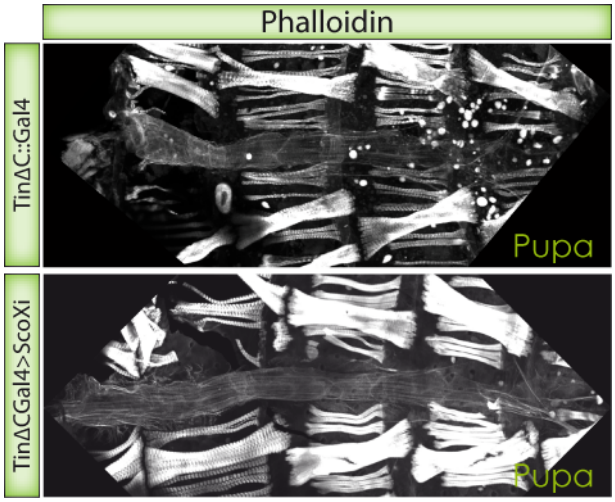

Figure 17. Cardiac specific *ScoX* knockdown does not disrupt pupal cardiac development.
Fluorescent-phalloidin staining from *TinCΔ4::G4* and *TinCΔ4G4>ScoXi* pupae hearts.

4. COX deficiency leads to an increased production of reactive oxygen species

We had already determined that heart specific ScoX knockdown causes mitochondrial dysfunction due to a decline in complex IV activity. Increased levels of reactive oxygen species (ROS) are a source of cellular stress (Yang et al. 2010) and are often associated with mitochondrial dysfunction. Mitochondrial respiration is the main

source of ROS in all eukaryotic cells, with two major superoxide producing sites: complex I and III of the ETC (St-Pierre et al. 2002). In order to determine whether reduced COX activity leads to an increased ROS production, we analyzed ROS levels in hearts from control and from 1- and 2-week-old *TinCΔ4-Gal4>ScoXi* flies using dihydroethidium (DHE) as an indicator (Figure 18). Hearts from *TinCΔ4-Gal4>ScoXi* displayed significantly increased ROS levels compared with control at both tested ages. Note that 2-week-old *ScoX* down-regulated cardiomyocytes displayed higher levels of DHE staining than did 1-week-old flies.

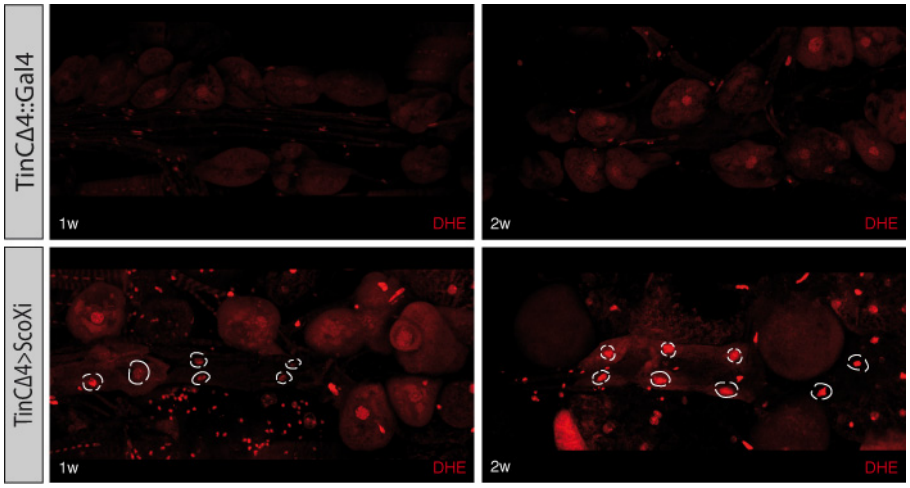

Figure 18. Cardiac-specific *ScoX* knockdown induces oxidative stress.
DHE staining in hearts from *TinCΔ4::G4* and *TinCΔ4G4>ScoXi* 1and 2-week-old flies. Cardiomyocyte nuclei are encircled in white.

5. ScoX cardiomyopathy is p53-dependent

p53 is a tumor suppressor with a central role in cancer development, apoptosis, senescence and differentiation (Vogelstein et al. 2000). Furthermore, a number of studies have implicated p53 in different types of cardiomyopathy (Birks et al. 2008; Nakamura et al. 2012). p53 also has been shown to have an important role in cellular stress responses regulating metabolic pathways like glycolysis (Mathupala et al. 2001; Ruiz-Lozano et al. 1999) and OXPHOS (Matoba et al. 2006). Interestingly, they found that in stress response, *p53* directly regulates aerobic respiration through the modulation of *Sco2*. *dp53*, the *Drosophila* homolog of *p53*, shares structural and functional properties with mammalian p53 (Jin et al. 2000; Ollmann et al. 2000). Given that p53 regulates stress response through the modulation of *Sco2* expression and since it is associated to the development of various cardiomyopathies, we speculated that *dp53* might also have a role in *D.*

melanogaster stress response and heart function. We reasoned that since in *ScoX* knockdown hearts OXPHOs activity is partially compromised, leading to an upregulation activation of the glycolytic pathway and possibly to higher lactic acid production, a p53-mediated stress response might be involved.

5.1. Overexpression of *dp53* in *ScoX* knockdown hearts

In order to address this question we measured *dp53* mRNA relative levels by qRT-PCR in hearts from control and *TinCΔ4-Gal4>ScoXi* at 1week old flies. We found that *dp53* transcript levels were significantly increased in *TinCΔ4-Gal4>ScoXi* (Figure 19 A). Then, to further investigate the role of dp53 in the heart degeneration observed in *ScoX* knockdown animals we next overexpressed dp53 in *TinCΔ4-Gal4>ScoXi* flies. Fluorescent microscopy analyses revealed that although cardiac dp53 overexpression itself caused a certain level of heart degeneration, *ScoX* knockdown hearts overexpresing dp53 exhibited a dramatic degeneration of the heart tube with a complete absence of myocardial myofibrils and only a few longitudinal myofibrils remaining in the dorsal vessel in the animals displaying the stronger phenotypes (Figure 19 B).

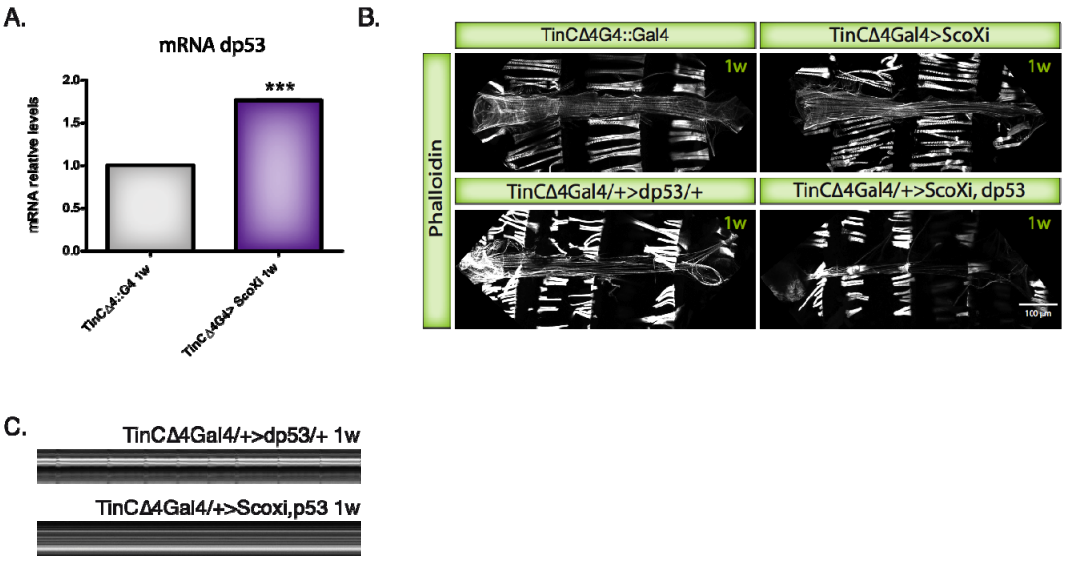

Figure 19. Cardiac-specific *Scox* knockdown induces p53 dependent heart degeneration.
(A) dp53 mRNA expression levels in *TinCΔ4::G4* and *TinCΔ4G4>ScoXi* hearts normalized to *RPL0* and relative to *TinCΔ4::G4*. Values are means ± S.E. n= 4. ***=p<0.001.
(B) Fluorescent-phalloidin staining from 1 week old adult *TinCΔ4::G4*, *TinCΔ4G4>ScoXi*, *TinCΔ4Gal4/+>dp53/+* and *TinCΔ4Gal4/+> ScoXi,dp53* hearts.
(C) Representative M-Mode traces (10s) from high-speed movies of 1 week old semi-intact flies.

To asses a functional relationship between dp53 and *ScoX* interference we then examined the cardiac physiology of 1-week-old *TinCΔ4-Gal4/+>ScoXi,dp53* flies. M modes revealed no heartbeat in *TinCΔ4-Gal4/+>ScoXi,dp53* flies. Consistent with the phenotype detected by fluorescence microscopy, overexpression of dp53 itself affects heart function at 1-week (Figure 19 C).

5.2. Overexpression of *dp53* in *Surf1* knockdown hearts

In order to rule out that the observed phenotype is not due to a general stress response triggered by mitochondrial malfunction but to a specific genetic interaction between *ScoX* down-regulation and dp53, we overexpressed dp53 in the fly heart together with a *TinCΔ4-Gal4* driven *Surf1* RNAi. Surf1 is a COX assembly factor involved, like ScoX, in the early steps of complex IV assembly. Mutations in the human *Surf1* gene cause Leigh syndrome, the most common infantile mitochondrial encephalopathy, with isolated complex IV deficiency (Tiranti et al. 1998; Zhu et al. 1998). It has been previously reported that knockdown of *Surf1* gene expression in *Drosophila melanogaster* causes COX deficiency, with nervous system involvement and developmental arrest (Zordan et al. 2006). First, we characterized the effects of heart specific *Surf1* knockdown on adult heart myofibrillar structure. *TinCΔ4-Gal4>Surf1i* animals show moderate levels of interference and 1-week-old animals displayed a similar, but milder, cardiac phenotype as that observed for *ScoX* knockdown (Figure 20 A-B).

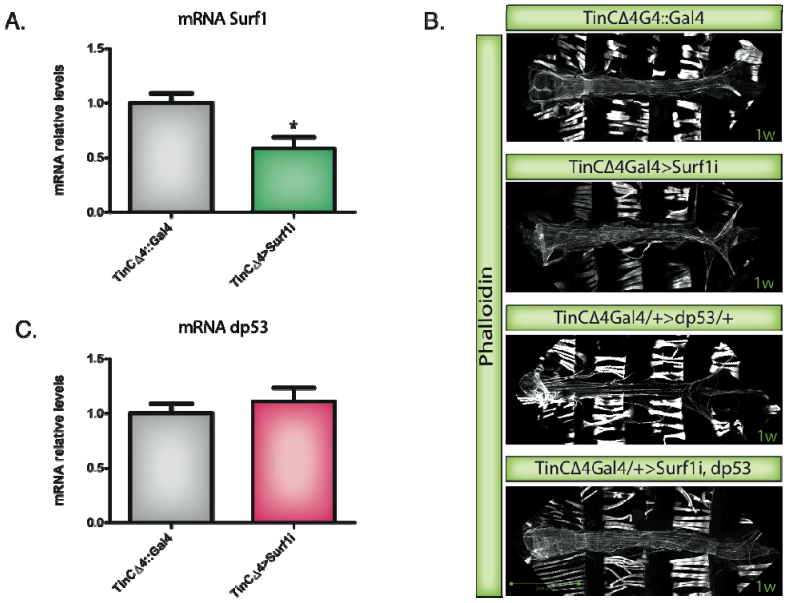

Figure 20. *dp53* expression in *Surf1* knockdown hearts does not affect heart structure.

(A) *Surf1* mRNA expression levels normalized to *RPL0* and relative to *TinCΔ4::G4*. Values are means ± S.E. n= 6-7. *=p<0.05.
(B) Fluorescent-phalloidin staining from 1 week old adult *TinCΔ4::G4*, *TinCΔ4G4>Surf1i, TinCΔ4Gal4/+>dp53/+ and TinCΔ4Gal4/+> Surf1i,dp53* hearts.
(C) *dp53* mRNA expression levels normalized to *RPL0* and relative to *TinCΔ4::G4*. Values are means ± S.E. n= 6-7.

Then, we tested whether dp53 overexpression in *TinCΔ4-Gal4>Surf1i* animals would cause the same cardiac degeneration observed in *TinCΔ4-Gal4/+>ScoXi,dp53* flies. Intringuingly, dp53 overexpression did not exacerbate myofibrillar disarray found in 1-week-old *TinCΔ4-Gal4>Surf1i* flies. On the contrary, it rescued the heart structure phenotype caused by *Surf1* RNAi knockdown (Figure 20 B). Furthermore, we found no increase in *dp53* transcript levels in *TinCΔ4-Gal4>Surf1* hearts from 1-week-old flies (Figure 20 C).

Taken together, these results demonstrate that dp53 and ScoX interact genetically, strongly suggesting that dp53 plays an important role in the development of *ScoX* knockdown induced cardiomyopathy.

6. Cardiac specific *ScoX* knockdown induces apoptosis

Given that apoptosis (Programmed cell death) and the pro-apoptotic function of dp53 is well conserved in *Drosophila* (Brodsky et al. 2004; Ollmann et al. 2000), the preceding experiments strongly suggest that *ScoX* knockdown might induce apoptosis through dp53 in cardiomyocytes.

6.1. Measurement of pro-apoptotic genes mRNA levels

In order to ascertain if apoptosis is activated in *TinCΔ4-Gal4>ScoXi* heart tubes, and since in response to stress dp53 controls cell death through the Reaper-Hid-Grim network (Brodsky et al. 2000; Chen et al. 1996; Fan et al. 2010; Grether et al. 1995a), we next tested the relative mRNA levels of *Reaper*, *Hid* and *Grim* by qRT-PCR. We observed a significant increase in *Reaper*, *Hid* and *Grim* RNA expression levels in heart tubes of *TinCΔ4-Gal4>ScoXi* at 1- and 2-week-old flies (Figure 21) demonstrating the activation of the apoptotic pathway in *ScoX* knockdown flies.

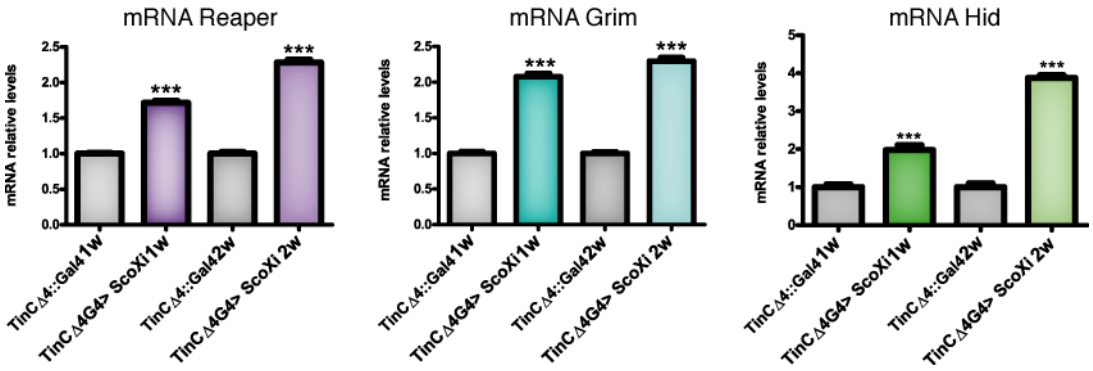

Figure 21. Cardiac-specific knockdown of ScoX induces the overexpression of pro-apoptotic genes.c
(A) *Reaper, Grim* and *Hid* mRNA expression levels normalized to *RPL0* and relative to *TinCΔ4::G4*. Values are means ± S.E. n= 5-8. ***=p<0.001.

6.2. TUNEL staining in *Scox* RNAi knockdown hearts

To support further the idea that apoptosis might be at the root of the observed heart degeneration we examined the predicted apoptotic response by TUNEL staining in hearts at 1- and 2-week-old *ScoX* knocked down flies. As expected, control hearts showed no TUNEL labeling in neither 1- nor 2-week-old animals (Figure 22 C 1-3). On the contrary, clear labelling was observed in 1-week-old *TinCΔ4-Gal4>ScoXi* hearts, with markedly stronger staining after 2 weeks (Figure 22).

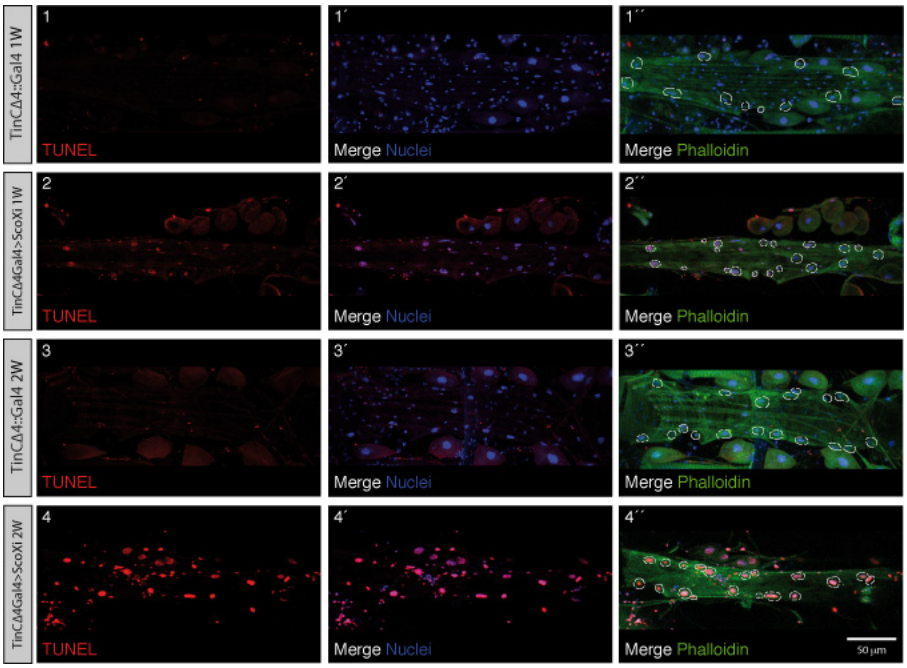

Figure 22. Cardiac-specific knockdown of *ScoX* induces apoptosis.
TUNEL (red), Dapi (blue) and Alexa-Fluor-phalloidin (green, merge) stainings of *TinCΔ4>ScoXi* and *TinCΔ4::G4* 1 and 2-weeks old flies. Cardiomyocytes nuclei are encircled in white.

6.3. *Reaper* overexpression

In order to confirm whether apoptosis was responsible for the heart structural degeneration observed in *TinCΔ4-Gal4>ScoXi* flies, we overexpressed the pro-apoptotic gene *Reaper* in a *TinCΔ4-Gal4>ScoXi* background and analyzed heart structure by phalloidin staining. Fluorescent imaging revealed that overexpression of *Reaper* in 1-week *TinCΔ4-Gal4>ScoXi* hearts resembled the collapsed phenotype observed in 2-week old cardiac-specific *Scox* knockdown flies (Figure 23 A-B).

These data clearly demonstrate that *ScoX* knockdown resulted in an activation of the *Drosophila* apoptotic pathway, thus inducing cell death.

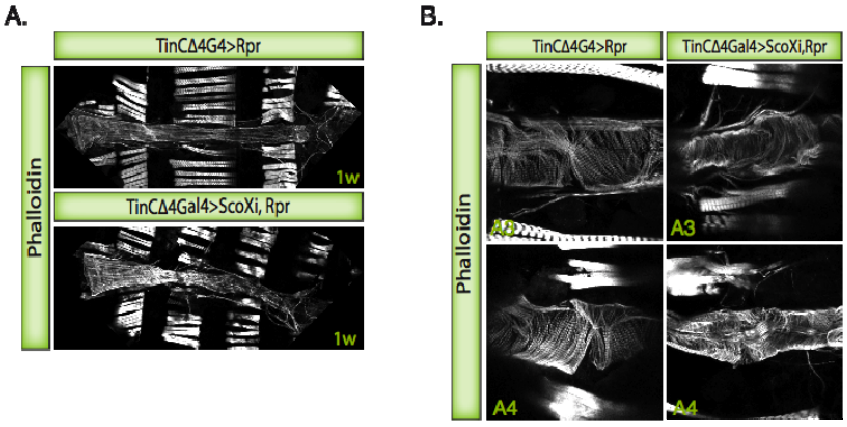

Figure 23. *Reaper* expression in *ScoX* knockdown hearts exacerbates degeneration. **(A)** Confocal images of 2-week-old *TinCΔ4::G4>Rpr* and *TinCΔ4G4>ScoXi, Rpr* adult hearts at 10X magnification and **(B)** of the A3 and A4 at 25X optical magnification (2X ZOOM). Adult hearts are stained with Alexa Fluor 594 phalloidin.

7. Blockage of dp53 activity rescues SCOX cardiomyopathy

7.1. Overexpression of a dominant negative form of *dp53* rescues heart dysfunction and heart structure phenotype

The fact that *ScoX* downregulation triggers dp53-mediated apoptosis, together with the strong degeneration observed in those flies (which is largely worsened by dp53 overexpression) lead us to hypothesize that the blockage of dp53 pathway might impede apoptosis triggering and rescue cardiac function. Therefore, we decided to use a dominant-negative form of dp53 ($dp53^{DN}$) to abrogate p53 activity. M-Modes from 2-week-old *TinCΔ4-Gal4/+>ScoXi*, $dp53^{DN}$ hearts exhibited regular rhythmic contractions

and an average heartbeat length, while dp53^{DN} cardiac overexpression itself did not affect heart function (Figure 24 A, compare to Figure 15 A).

We then examined cardiac physiology in dp53^{DN} overexpressing animals. *TinCΔ4-Gal4/+>ScoXi, dp53^{DN}* significantly rescued *TinCΔ4-Gal4>ScoXi* cardiac dysfunction in 2-week old flies (Figure 24 B). The presence of a *UAS::GFP* in a *TinCΔ4-Gal4/+>ScoXi/+* genetic background almost completely rescues the *ScoX* downreguled physiological phenotype (Figure 24 B), rising the possibility that the observed rescue was due to a lower level of interference rather than a to a blockage of the p53 pathway. However, animals harboring one *TinCΔ4-Gal4* copy driving the expression of both GFP and ScoX (*TinCΔ4-Gal4/+>ScoXi, GFP*) still displayed a longer diastolic interval than did controls or *TinCΔ4-Gal4/+>ScoXi, dp53^{DN}*, showing that this rescue was not complete (Figure 24 B).

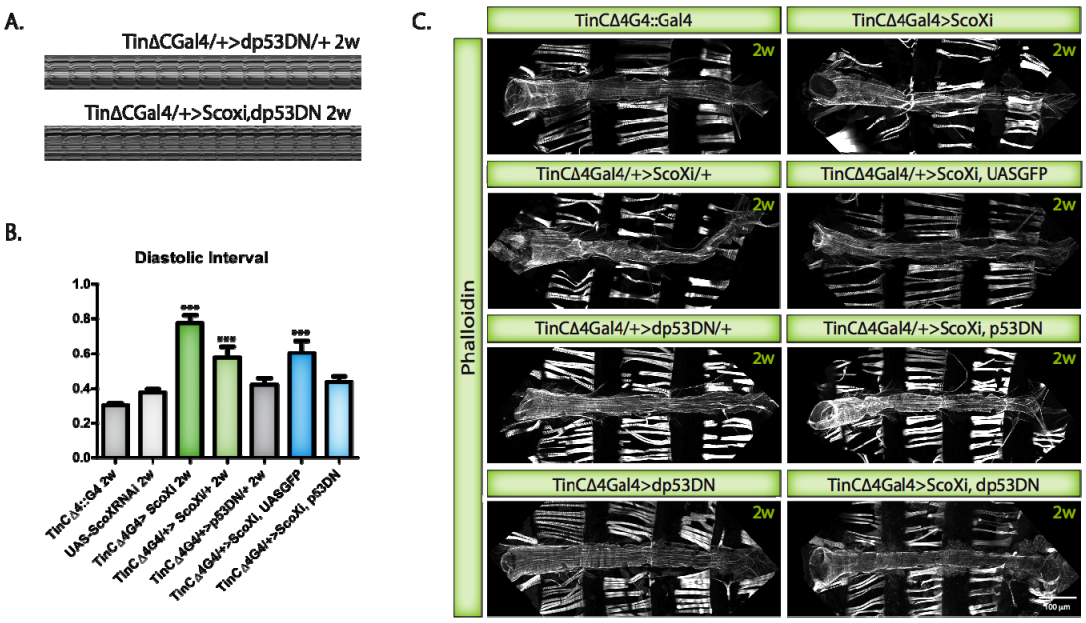

Figure 24. Lack of *dp53* activity rescues Scox cardiomyopathy.
(A) Representative M-Mode traces (10s) from high-speed movies of 1 week old semi-intact flies.
(B) Diastolic interval in 2-week-old hearts from semi-intact flies. Differences are relative to the *TinCΔ4::Gal4*. Values are means ± S.E. n= 20-40 flies. Error bars indicate SEM. ***=p<0.001.
(C) Fluorescent-phalloidin staining from 2 week old adult hearts at 10X magnification. Adult hearts are stained with Alexa Fluor 594 phalloidin.

To further rule out the possibility the rescue observed above was due to a lower level of interference rather than a to a blockage of the p53 pathway, we increased the Gal4 dosage so the ratio Gal4/UAS insertion was 1/1. We next examined cardiac structure

using phalloiding staining to visualize the actin organization within the myofibrils in 2-week old flies. Fluorescent imaging revealed that the morphological defects observed in *TinCΔ4-Gal4>ScoXi* hearts were fully rescued by the overexpression of $dp53^{DN}$. Note that even if *TinCΔ4-Gal4/+>ScoXi, UASGFP* rescued cardiac performance it did not completely restore heart structural defects (figure 24 C). On the contrary, *TinCΔ4-Gal4>ScoXi*, $dp53^{DN,}$ carrying two driver copies, exhibited a perfectly aligned circumferential myofibrillar arrangement within the cardiomyocytes like controls. Again, overexpression of $dp53^{DN}$ alone did not affect heart morphology (Figure 24 C).

Under higher magnification, A3 and A4 segment analysis further confirmed our results. Cardiac-specific overexpression of $dp53^{DN}$ fully rescued the severe morphological defects found in *TinCΔ4-Gal4>ScoXi* (Figure 25). It should be noted, that even in the presence of a *UAS-GFP*, just one copy of *UAS-ScoXRNAi* insertion, *TinCΔ4-Gal4/+>ScoXi/+* and *TinCΔ4-Gal4/+>ScoXi, UASGFP*, was enough to cause significant morphological defects (Figure 25).

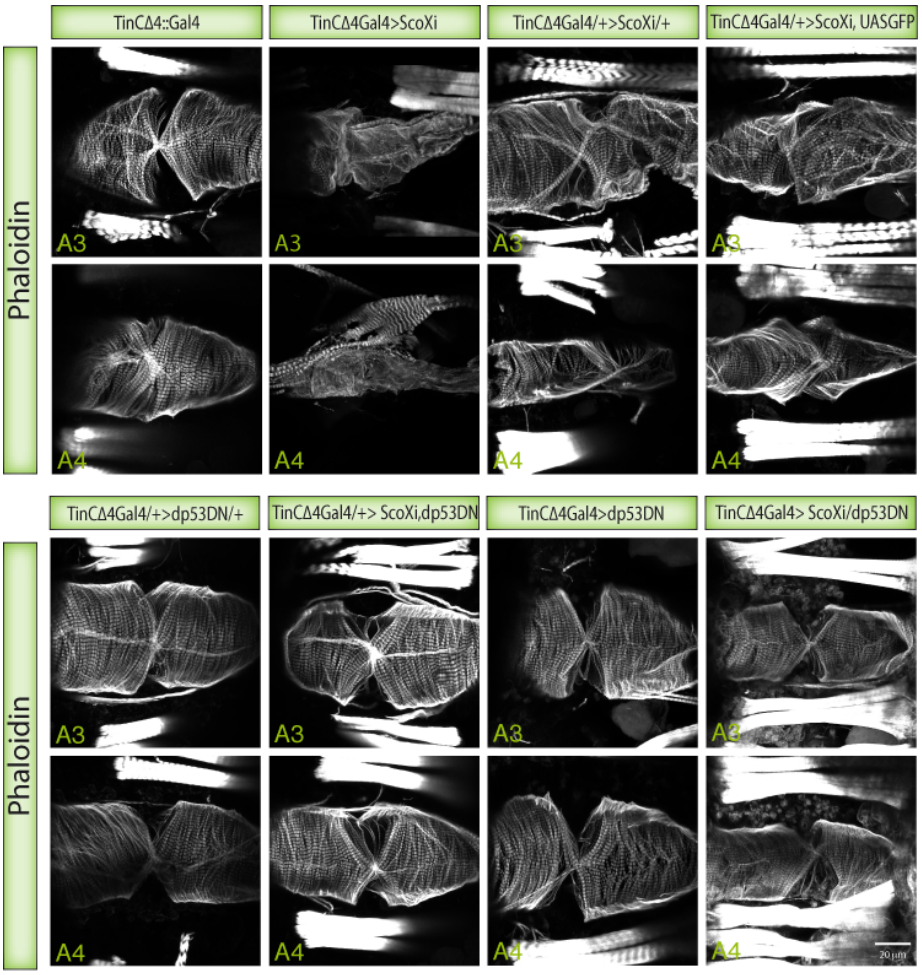

Figure 25. Lack of dp53 activity rescues *Scox* structure phenotype
Fluorescent-phalloidin staining of 3rd (A) and 4th (A4) abdominal segment of the dorsal vessel from 2 week old adult hearts.

Even more, absence of p53 prevented cardiomyopathy, since heart specific *ScoX* interference in a p53 null background results in a complete absence of degeneration (Figure 26) further emphasizing p53's role in the development of the cardiomyopathy.

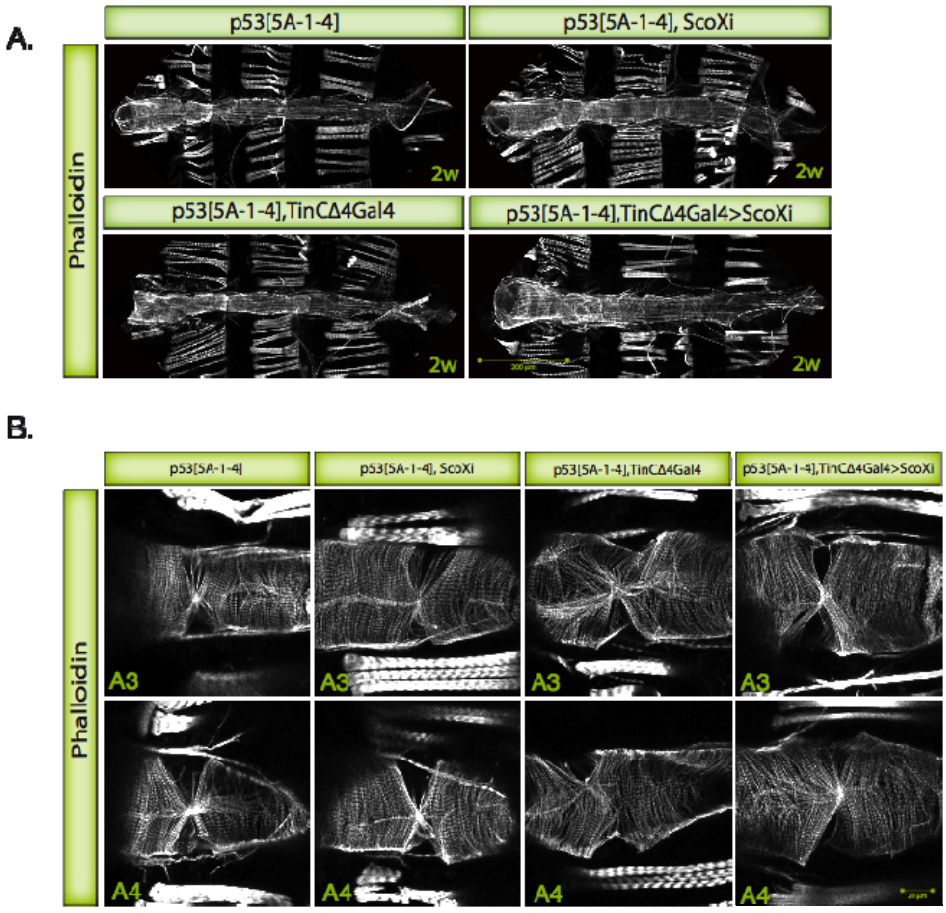

Figure 26. *ScoX* interference in a p53 null background rescues degeneration.
(A) Fluorescent-phalloidin staining from 2 week old adult *dp53[5A-1-4], dp53[5A-1-4] ScoXi, dp53[5A-1-4] TinCΔ4G4* and *dp53[5A-1-4] TinCΔ4>ScoXi*.
(B) Detail from abdominal segments 3 and 4.

7.2. Overexpression of a DN form of *dp53* rescues metabolic switch

We have previously shown that cardiac specific interference of *ScoX* resulted in significantly altered glucose metabolism. It has been shown that, in mice, p53 is involved in the maintenance of the metabolic homeostasis (reviewed in (Olovnikov et al. 2009). Given the full rescue observed in *TinCΔ4-Gal4/+>ScoXi*, $dp53^{DN}$ we decided to assess

whether *dp53*^{DN} overexpression restores the enhanced glycolysis observed in *TinCΔ4-Gal4>ScoXi* flies. Interestingly, analysis of the selected mRNA transcripts levels by qRT-PCR showed that the overexpression of *dp53*^{DN} restores PFK, IMPL3, and PDK expression to its normal levels (Figure 27 A-C). Most importantly, *ScoX* knockdown is slightly affected by *dp53*^{DN} overexpression (Figure 27 D), further demonstrating that blockage of *dp53* activity results in a complete rescue of cardiac function, heart structure and altered glycolytic metabolism observed in *TinCΔ4-Gal4>ScoXi* flies. Furthermore, our results demonstrate that the *Drosophila p53* homolog plays an important role in the regulation and maintenance of metabolism homeostasis as it does in mammals (Berkers et al. 2013; Maddocks and Vousden 2011).

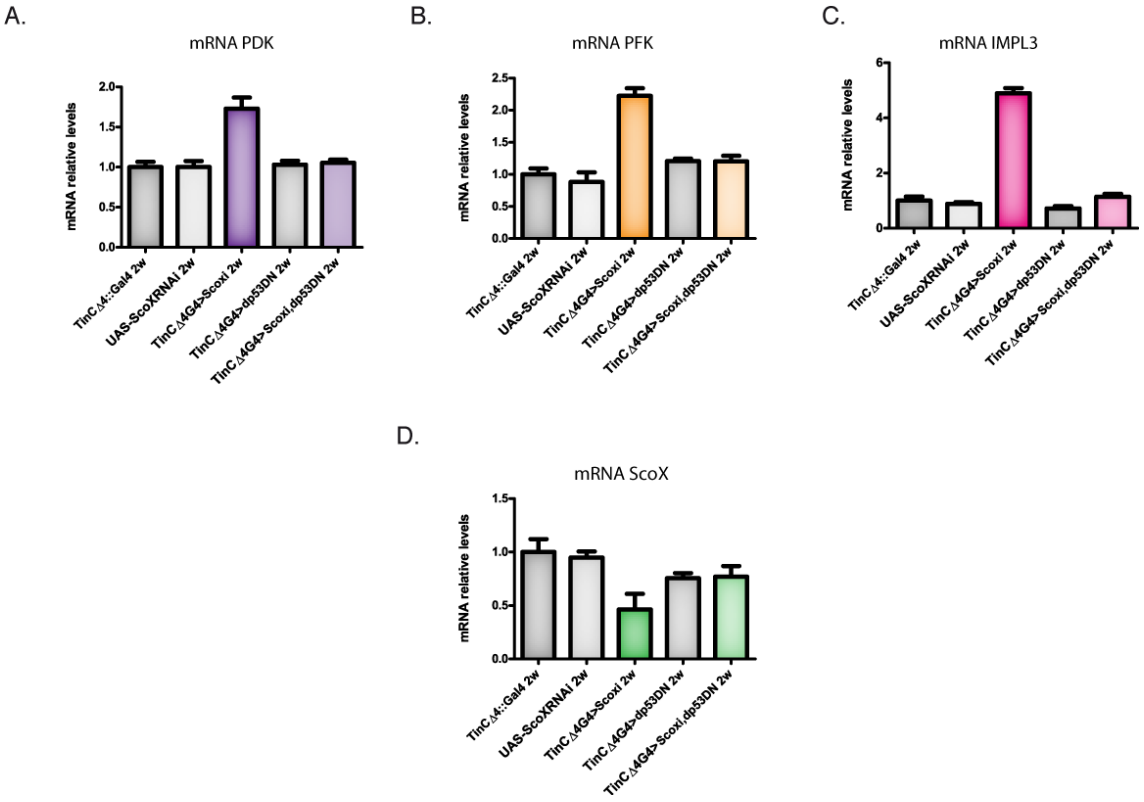

Figure 27. Lack of dp53 activity rescues *Scox* metabolic switch.
(A-D) mRNA expression levels *of PDK, PFK, IMPL3* and *ScoX* in hearts from 2-week old *TinCΔ4::G4, UAS-ScoXi, TinCΔ4G4>ScoXi, TinCΔ4G4>p53*^{DN} and *TinCΔ4G4>ScoXi,p53*^{DN} flies normalized to *RPL0* and relative to *TinΔC::G4*. Values are means ± S.E. n= 6-10. ***p < 0.001.

8. Inhibition of apoptosis rescues structural degeneration

Next, we asked whether inhibition of apoptosis would block cell death and therefore rescue the structural degeneration observed in *TinCΔ4-Gal4>ScoXi*

cardiomyocytes. To address this question we overexpressed the baculovirus caspase inhibitor *p35* (Hay et al. 1994) and the *Drosophila* inhibitor of apoptosis *DIAP1* (Hay et al. 1995; Wang et al. 1999) in a *TinCΔ4-Gal4>ScoXi* background. Cardiac myofibrillar structure was assessed by inmunofluorescence microscopy. Overexpression of either *p35* or *DIAP1 TinCΔ4-Gal4>ScoXi* hearts almost completely rescued the cardiac structure phenotype in 2-week-old (Figure 28 A). Under high magnification, myocardial cells exhibited a normal myofibrillar pattern, with an almost complete rescue of the phenotype when we overexpressed p35 and a milder one when we overexpressed *DIAP1* (Figure 28 B). Thus, these data show that inhibition of apoptosis rescues the myofibrilar structure degeneration observed in *TinCΔ4-Gal4>ScoXi* flies.

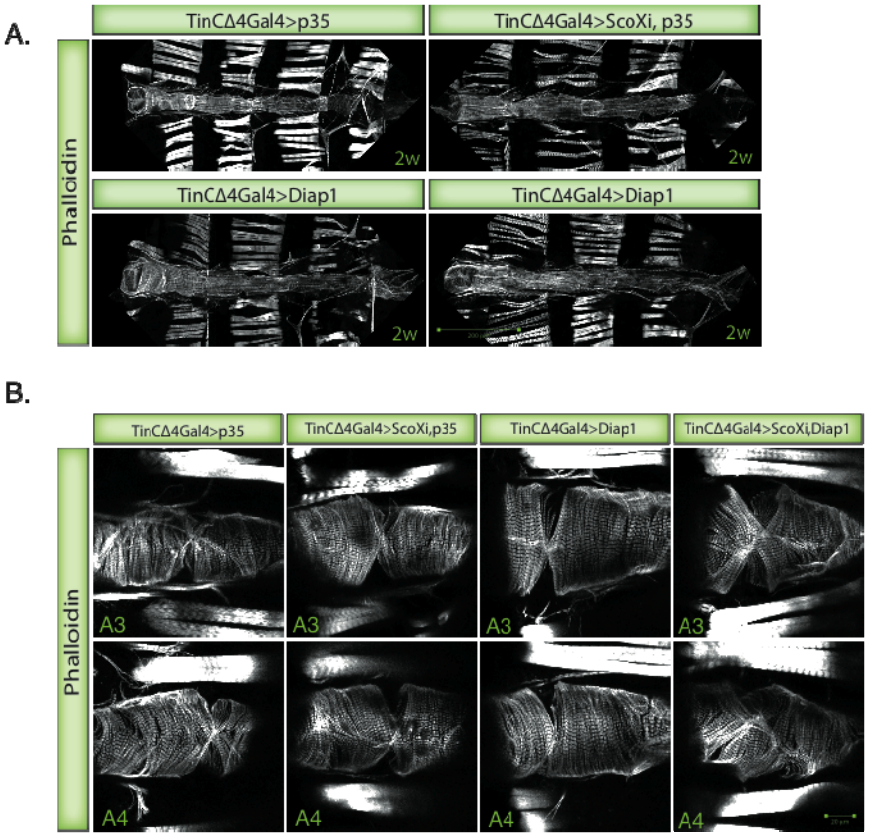

Figure 28. *p35* or *DIAP1* expression in *ScoX* knockdown hearts almost rescues degeneration.
(A) Fluorescent-phalloidin staining from *TinCΔ4::G4>p35*, *TinCΔ4::G4>ScoX,p35*, *TinCΔ4::G4>DIAP1* and *TinCΔ4G4>ScoXi, DIAP1* hearts.
(B) Detail from abdominal segments 3 and 4 depicted above.

9. *Sco2*^{KIKO} mice undergoes apoptosis

Thus, if partial loss of *ScoX* in *Drosophila* leads to apoptosis as a final consequence, and since its function is conserved in mammals, we wondered whether our results in *Drosophila* could be extended to mammalian system. Although unfortunately there are no *Sco1* KO mice available, a *Sco2*^{KIKO} human disease mouse model has been recently developed (Yang et al. 2010). *Sco2*^{KIKO} mice harbor both a *Sco2* knock-out allele and the *Sco2* knock-in E129K allele. The mice E129K allele corresponds to the E140K mutation found in almost all human patients (Mobley et al. 2009; Yang et al. 2010).

Consistent with what it has been observed in patients with SCO2 deficiency, *Sco2*^{KIKO} mice model showed motor impairments and biochemical and functional defects. In contrast to human, the reduction in COX activity in *Sco2*^{KIKO} mice was less severe in muscle than in other tissues, such as liver, and was accompanied by an unexpected defect in complex III activity (Yang et al. 2010). Although *Sco2*^{KIKO} mice do not present all the features of the human disease, they are viable and fertile, which makes it an excellent model for the search of pharmacological therapies. When *Sco2*^{KIKO} mice were treated with AICAR, an AMPK agonist, it leads to a partial rescue of COX activity with motor improvement (Viscomi et al. 2011). Hence, we decided to use *Sco2*^{KIKO} mice to validate our *Drosophila* data in a mammalian model system. *Sco2*^{KIKO} liver and skeletal muscle, but not tissue from wt animals, had massive apoptosis as shown by TUNEL staining (Figure 29). Furthermore, *Sco2*^{KIKO} liver seemed to have more apoptotic cells than did muscle, in concordance with the lowest complex IV activity value observed in *Sco2*^{KIKO} liver compared with other examined tissues (Yang et al. 2010). We concluded that, as in *Drosophila,* partial loss of Sco2 in mice function induces apoptosis, further validating our findings.

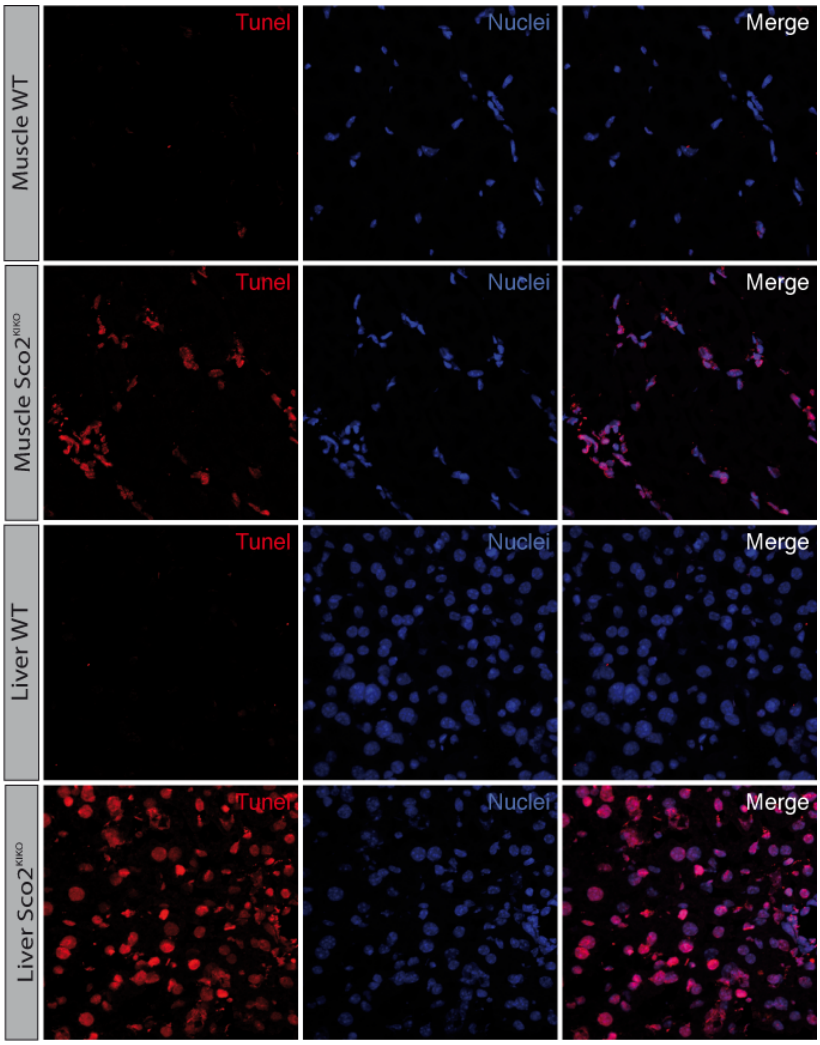

Figure 29. *Sco2*^{KIKO} mice undergo apoptosis in liver and skeletal muscle.
TUNEL (red) and Dapi (blue) stainings of Skeletal muscle and liver from wt and *Sco2*^{KIKO} 6-months-old mice. n=3.

DISCUSSION

Mitochondrial diseases due to defects in cytochrome c oxidase (COX) are one of the most frequent respiratory chain disorders in humans. Patients displaying these pathologies present extreme heterogeneity of clinical manifestations with an onset varying from neonatal to adult life, and multiple organ involvement such as brain, skeletal muscle and heart. This heterogeneity makes considerably difficult the understanding of the pathophysiology of these diseases. Pathogenic mutations in human *SCO1* and *SCO2* have been reported to cause hypertrophic cardiomyopathy among other clinical symptoms (Leary et al. 2013; Papadopoulou et al. 1999). However, the molecular mechanisms underlying this cardiac dysfunction have yet to be elucidated.

Here, we present the first cardiac-specific animal model to study human Sco1/2 mediated cardiomyopathy. Cardiac-specific knockdown of *ScoX* results in severe dilated cardiomyopathy due to mitochondrial dysfunction with a concomitant metabolic switch from glucose oxidation to glycolysis and increased ROS levels, leading to p53 dependent cell death as fatal outcome. Moreover, partial loss of Sco2 function induces apoptosis in liver and skeletal muscle in a $Sco2^{KIKO}$ mouse model. We also report a new role for dp53 in homeostasis regulation and maintenance of metabolism, a function conserved in mammals**.**

1. Drosophila melanogaster ScoX

ScoX is a conserved complex IV assembly factor presenting homology with the human *Sco1* and *Sco2* genes (Porcelli et al. 2010). Ubiquitious ScoX knockdown in *Drosophila* is lethal in third instard larva, displaying a *spargel* phenotype and a severe COX deficiency thus phenocoping its loss of function (Figure 10) (Porcelli et al. 2010). Furthermore, this reduction in COX activity is in concordance to that observed in patients displaying mutations in either *Sco1* or *Sco2* genes (Jaksch et al. 2001; Leary et al. 2013).

Although the role of Sco proteins as copper metallochaperones is largely documented, they have been shown to be involved in the maintenance of cellular copper homeostasis, presumably by controlling cellular copper export (Leary et al. 2007; Leary et al. 2009a), the molecular mechanism underlying the cardiac dysfunction caused by mutations in human *Sco1* and *Sco2,* have yet to be elucidated (Leary et al. 2013; Papadopoulou et al. 1999). In the present study, we got advantage of the genetic versatility of the *Drosophila* model to investigate ScoX loss of function in the heart. A cardiac-specific knockdown of *ScoX* by 50% at the RNA level was sufficient to cause a deficiency in COX activity suggesting that the knockdown might be more efficient at the protein activity level (Figure 12).

In order to maintain the glycolytic flux and compensate for the fall in ATP production caused by COX deficiency, *ScoX*-knockdown hearts displayed a compensatory upregulation of glycolysis, partial blockage of the Krebs cycle, and increased *Impl3* expression (the ortholog of the mammalian LDH), most probably resulting in lactic acidosis (Figure 13). Interestingly, previous studies in patients and rat models, have shown that mitochondrial dysfunction is associated with abnormalities in cardiac function and changes in energy metabolism, resulting in glycolysis optimization and lactic acidosis (Nascimben et al. 2004) reviewed in (Honzik et al. 2012; Schiff et al. 2011).

2. *ScoX* knockdown induces dilated cardiomyopathy in Drosophila.

Since all *Sco2* patients die from cardiac insufficiency, we assessed cardiac function in *Scox* knockdown flies using functional imaging. This experimental approach allows us to analyze the physiological impact of *ScoX* interference in heart function and disease. In contrast to what was observed in the mouse *Sco2*$^{KO/KI}$ model, where no evidence of cardyomiopathy was described (Yang et al. 2010), cardiac-specific *ScoX* knockdown resulted in a severe impairment of cardiac function. Flies developed dilated cardiomyopathy resembling cardiomyopathies caused by mitochondrial fusion defects in flies (Dorn et al. 2011). *ScoX* knockdown also caused a significant reduction of FS as well as prolonged DIs (Figure 15) accompanied by an age-associated cardiac myofibril disorganization (Figure 16). Furthermore, these cardiac defects are likely caused by a degenerative process most likely due to an adult-onset mitochondrial dysfunction rather than to a developmental defect, since pupae heart structure remained unaffected (Figure 17).

3. COX deficiency in fly heart results in ROS production

The electron transport chain (ETC), within the mitochondrial inner membrane is the main site of ROS production in the cell (reviewed in (reviewed Koopman et al. 2010). Many neurodegenerative diseases and the aging process has been link to mitochondrial dysfunction that triggers excessive oxidative stress production, mostly due to an impairment of the ETC (reviewed in M. T. Lin and Beal 2006). Previous studies in human cells have shown that *Sco2 -/-* cells display an increased ROS formation with oxidative DNA damage (Sung et al. 2010).

We found that cardiac-specific knockdown of *ScoX* induces oxidative stress production (Figure 18). However, we cannot distinguish whether this increase in free radical accumulation arises from the mitochondria, due to a loss of COX function, or if it comes from non-mitochondrial sources as a consequence of the loss of cellular homeostatic control, as it has been shown in yeast (Leadsham et al. 2013) or in a neuro-specific COX-deficient Alzheimer disease mouse model (Fukui et al. 2007).

4. *dp53* is involved in cardiomyopathy development

The p53 tumor suppressor, is a well described transcription factor known for its participation in cancer development, DNA repair, cell cycle, autophagy, necrosis and apoptosis and senescence (reviewed in Berkers et al. 2013; Vousden and Prives 2009). Beyond these functions, increasing evidences of new roles for *p53* have been reported, including its ability to regulate both glycolyisis and OXPHOS, through the modulation of *Sco2* (Bensaad et al. 2006; Mathupala et al. 2001; Matoba et al. 2006; Ruiz-Lozano et al. 1999). Furthermore, the contribution of p53 in cardiovascular disorders has been demonstrated (Birks et al. 2008; Nakamura et al. 2012; Sano et al. 2007; Shimizu et al. 2012).

In this scenario and since p53 activation in response to stress signals, like increased oxidative stress or latic acidosis, is well documented (Johnson et al. 1996; Olovnikov et al. 2009; Zhuang et al. 2012), we hypothesized that *dp53* might play an important role in the cellular response to ScoX loss of function. We found that *p53* expression was upregulated in cardiac-specific *ScoX* knockdown hearts (Figure 18A). Moreover, the overexpression of *dp53* in *TinCΔ4-Gal4>ScoXi* flies dramatically affects heart structure and physiology, as demonstrated by the lack of heart beat in *TinCΔ4-Gal4/+>ScoXi,dp53* flies **(**Figure 19 B-C). Interestingly, we observed that dp53 overexpression in a cardiac-specific *Surf1* knockdown animals, displying a heart phenotype similar to that observed in *ScoX* interfered flies, rescued the heart structure phenotype (Figure 20), further stressing the specificity of dp53 involvement in the development *ScoX* induced cardiomyopathy.

5. *dp53* contributes in the development of *ScoX* mediated cardiomyopathy

The strong degeneration observed in *TinCΔ4-Gal4>ScoXi* flies together with the almost complete disappearance of the fly heart when p53 is overexpressed in these flies, strongly suggests that the apoptotic pathway might be activated in *TinCΔ4-Gal4>ScoXi* hearts.

Interestingly, it has been recently reported that Sco2 overexpression induces p53-mediated apoptosis in tumor xenografts and cancer cells (Madan et al. 2013).

Furthermore, in a previous report, it was shown that *Sco2* knockdown in p53 +/+ glioma cells, but not in those negative for it, sensitivized them to hypoxia-induced apoptosis in a p53 dependent manner and induced necrosis in tumors expressing wild-type *p53* (Wanka et al. 2012), further liking the *Sco2/p53* axe to cell death.

In *Drosophila*, heart specific *ScoX* knockdown induces dp53 mediated cell death in cardiomyocytes and it does so through the activation of the canonical apoptotic pathway since *dp53* target genes *Reaper*, *Hid* and *Grim* are upregulated (Figure 20-21). Moreover, Reaper over-expression in adult heart enhances the structural defects observed in *TinCΔ4-Gal4>ScoXi* flies (Figure 23). Consistent with the results in the fly heart, we found that skeletal muscle and liver from $Sco2^{KIKO}$ mice undergoes massive cell death as well (Figure 29). These data suggest a conserved response to Sco2 loss of function mediated stress.

We provide evidence that *TinCΔ4-Gal4>ScoXi* hearts exhibit a partial loss of COX activity. Therefore, we hypothesize mitochondrial dysfunction not to be the only reason of the profound cardiomyopathy and phenotype observed. Moreover, in contrast to our results, prior evidence from vertebrate and invertebrate models has shown that a partial inhibition of mitochondrial respiration, promotes longevity and metabolic health due to a hormetic response (Lee et al. 2010; Liu et al. 2005; Rea et al. 2007). In fact, it has been recently shown that a mild interference in OXPHOS system activity in *Drosophila* IFMs preserves mitochondrial function, improves muscle performance, and increases life span through the activation of the mitochondrial unfolded pathway response and IGF/like signalling pathways (Owusu-Ansah et al. 2013).

Based upon these data, we speculate that cell death, rather than mitochondrial dysfunction itself, is likely to be the main reason for the profound heart degeneration detected in *TinCΔ4-Gal4>ScoXi* flies. Expression of a $dp53^{DN}$ in *ScoX* knocked-down hearts rescues cardiac dysfunction and degeneration and *TinCΔ4-Gal4>ScoXi*, *dp53* null animals display no apparent heart defects. Thus, we can attribute the observed rescue to a blockage of the p53 pathway (Figure 24-25).

Moreover, to further rule out that the observed rescue was due to a competition for the GAL4 among the two UAS insertions, we generated a *TinCΔ4-Gal4>ScoXi*, $dp53^{DN}$

line carrying two driver copies. Here, we find that the overexpression of *dp53^{DN}* restore the morphological defects observed in *TinCΔ4-Gal4>ScoXi* hearts. Thus, we can attribute the observed rescue to a blockage of the p53 pathway (Figure 23-24). Interestingly, *in vitro* silencing of p53 in cardiomyocytes prevented the mitochondrial damage and cardiomyocyte necrosis linked to monoamine oxidase- A (MAO-A) activation, further validating our findings (Villeneuve et al. 2013)

In accordance, inhibition of apoptosis by *p35* or *Diap1* over-expression resulted in an almost complete rescue of the *ScoX* knockdown morphological phenotype. Thus, even if inhibition of apoptosis can significantly prevent cardiac dysfunction significantly, it is not a full rescue (Figure 28). The inability of *p35* and *Diap1* to completely rescue the morphological phenotype suggests that in addition to apoptosis induction, *dp53* plays a key role in cardyomyopathy development. Nevertheless, it remains to be determined if the activation of *dp53,* and therefore cell death, is a side effect of COX dysfunction and loss of cellular homeostasis or if the partial lack of *ScoX* itself triggers *dp53* upregulation and apoptosis.

Inhibition of complex IV by a morpholino antisense oligomer against Surf1 and CoxVa in fish causes COX deficiency, organ development defect, activation of programmed cell death and cardiovascular dysfunction (Baden et al. 2007). Interestingly, apoptosis was just found in head region, neural tube and trunk but not in the heart of COX-deficient fish, even though they display a strong cardiac dysfuntion. In contrast to zebrafish, *Surf1* KO mice despite showing reduced COX levels and increased blood lactate levels, displayed increased longevity (Dell'agnello et al. 2007), enhanced neuroprotection and cognitive function even though presenting increased ROS generation and glucose metabolism (A. L. Lin et al. 2013). Since loss of *Surf1* in humans causes Leigh Syndrome (Tiranti et al. 1998; Zhu et al. 1998), these studies in the mice support the idea that *Surf1* might have additional roles, like its participation in Ca^{2+} homeostasis (Dell'agnello et al. 2007).

In this sense, it is tempting to hypothesize that ScoX might have another role independent from its function as a COX assembly factor, perhaps as redox sensor (J. C. Williams et al. 2005), and may act in conjunction with dp53 to accomplish this role. In this context, is worth mentioning that it has been recently shown that *Sco2* interference in

mammalian cells induces the relocalization of p53 from mitochondria to the nucleus (Zhuang et al. 2013). On the other hand, it may be also possible that the threshold of COX deficiency tolerable by the heart might be lower as compared with other tissues, reflecting a tissue-dependent phenomenon or a tissue-dependent role of *ScoX*. More work is necessary to test this hypothesis and try to understand how the partial lack of ScoX induces cell death through dp53.

5.1 dp53 plays a role in the maintenance of metabolic homeostasis.

We found that ScoX knockdown in the heart of *Drosophila* leads to a mitochondrial dysfunction, hence resulting in mitochondrial respiration deficiency and the promotion glycolysis as major ATP source (Figure 13). Most probably, as suggested by increased *Impl3* expression, pyruvate formed during glycolysis will be transformed to lactate resulting in lactic acidosis.

Since p53 has the ability to participate in different and apparently opposing groups of responses regarding metabolic homeostasis, we examined whether blockage of dp53 would have some impact in the altered glycolytic metabolism observed in heart-specific *ScoX* knockdown flies. Loss of p53 compromises cytochrome *c* oxidase function and supports a switch to glycolytic metabolism in both cultured cells and mouse models (Matoba et al. 2006). Here, we found the opposing response, where the overexpression of $dp53^{DN}$ restore glycolytic metabolism, rescuing the metabolic phenotype caused by the loss of ScoX (Figure 26). Based on current evidence, we propose a new role for *dp53* in the maintenance of metabolic homeostasis in *Drosophila*.

5.2. Connecting mitochondria and dp53 to cell death.

Mitochondria play multiple roles in apoptosis in many organisms (Kroemer and Reed 2000). Although the apoptotic machinery in *Drosophila* appears to be superficially similar to that of mammals, the role of mitochondria in *Drosophila* apoptosis is less well understood. As it have been mentioned, in mammalian systems, cyt c is released on MOMP. However, the role of cyt c in *Drosophila* apoptosis remains controversial since cyt c is not released in drug- or UV-induced apoptosis in fly cells (Dorstyn et al. 2004; Means et al. 2006; Zimmermann et al. 2002). On the other hand, *in vivo* data support a role for

cyt c in caspase activation during spermatogenesis and death of inter ommatidial cells (Arama et al. 2003; Arama et al. 2006).

Although the role of mitochondria in *Drosophila* apoptosis is still unclear there are some clear evidences suggesting that mitochondria play an important role in flies cell death as it does in mammals. In fact, it is known that Rpr, Hid and Grim localization in the mitochondria is essential to promote cell death (Claveria et al. 2002; Haining et al. 1999; M. R. Olson et al. 2003). Hid has a mitochondrial targeting sequence localized at its C-terminus (Haining et al. 1999) and it seems to be responsible for Reaper recruitment to mitochondria (M. R. Olson et al. 2003). The proapoptotic GH3 domain of Reaper, which is also found in Grim (Claveria et al. 2002) is required for mitochondrial localization and promotion of apoptosis. Moreover, it has been reported that expression of Reaper and Hid induce cyt c release into the cytosol of S2 cells and that under apoptotic stimulus, fly mitochondria undergoes Rpr, Hid and Drp1-dependent morphological changes and disruption during cell death (Abdelwahid et al. 2007). The participation of Drp1, a mitochondrial fission protein, in cell death shows a conserved role for mitochondria between flies and mammals which is also conserved in worms (Frank et al. 2001; Jagasia et al. 2005).

It is well established that in mammalian systems, in response to cellular stress, p53 protein translocates to mitochondria and triggers MOMP (Marchenko 2000; Mihara et al. 2003; Sansome et al. 2001). This, leads to the release of mitochondrial death factors like cyt c, AIF, SMAC/Diablo, and Omi/HTRA2 with the consequent activation of caspases (Danial and Korsmeyer 2004). In contrast to what it has been observed in mammals, release of mitochondrial factors does not appear to play a role in apoptosis in *Drosophila* S2 culture cells (Means et al. 2006). Therefore, MOMP is likely to be a consequence rather than a cause of caspase activation in *Drosophila* (Abdelwahid et al. 2007).

It has recently been proposed that p53 plays an essential role in mitochondrial permeability transition pore (mPTP) opening to induce necrotic cell death (Vaseva et al. 2012). Vaseva and collaborators propose that upon ROS stimulation, p53 translocates to the mitochondrial matrix where it binds cyclophilin D (CypD) to induce mPTP opening independent of proapoptotic Bcl-2 family members Bax and Bak. Furthermore, in contrast to the traditional view, where mPTP opening is triggered by Ca^{2+} overload accompanied by ROS generation and ATP depletion authors showed mPTP opening is independent of Ca^{2+} (reviewed in (Siemen and Ziemer 2013).

Apoptotic and regulated necrotic pathways have a number of common steps and regulatory factors among which is mPTP opening, which is thought to act through mitochondrial swelling and posterior delivery of necrotic factors (Nikoletopoulou et al. 2013). *Drosophila* mPTP has been recently characterized and, in contrast to what it has been described in mammals, mPTP activation it is not accompanied by mitochondrial swelling (von Stockum et al. 2011).

Thus, we hypothesize that dp53 induces mPTP opening to trigger cell death in cardiac-specific *ScoX* knockdown flies. In the absence of mitochondrial swelling, mPTP opening would result in apoptosis instead of necrosis as it has been shown in mammals. Moreover, apart from some evidence that suggests necrosis may play an important role in nurse cells during late oogenesis, no other necrotic events have been described in *Drosophila* so far (Jenkins et al. 2013).

Furthermore, even though *Drosophila* mPTP has been shown to be cyclosprin A (CsA)-insensitive *in vitro* (von Stockum et al. 2011), it has been recently shown that collagen XV/XVIII mutants, displaying mitochondrial dysfunction with severely attenuated ATP and enhanced ROS production, are improved by CsA administration (Momota et al. 2013). Interestingly, mice lacking collagenVI display mitochondrial structural alterations and spontaneous apoptosis. These defects are caused by mPTP opening and are normalized *in vivo* by CsA treatment (Irwin et al. 2003).

Future studies would be needed to get more insights into the relationship between dp53 and ScoX and into the mechanism that activates dp53 to trigger cell death. Our results may help to increase our understanding of Sco1 and Sco2-based cardiomyopathy and may represent a new target for therapeutic agents.

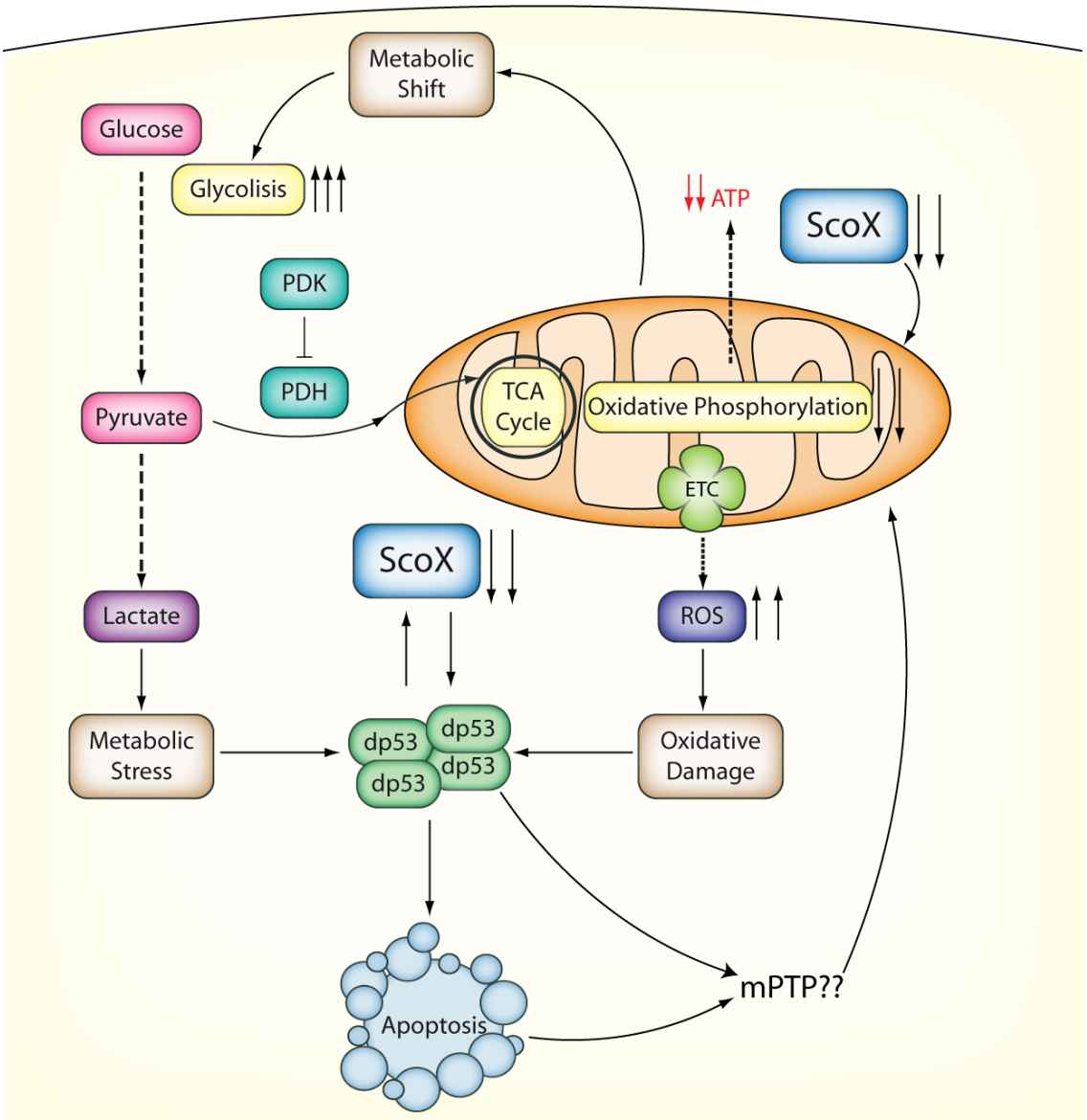

Figure 30. Schematic representation of our *ScoX* cardiomyopathy model.

In sum, we have generated the first animal model in *Drosophila melanogaster* for the study of human Sco1 and Sco2 mediated cardiomyopathy. Cardiac-specific knockdown of ScoX results in a metabolic switch from oxidative phosphorylation to glycolysis and cardiomyocyte cell death in a p53-dependent manner as response to loss of cellular homeostasis. Our data suggest a new role for ScoX independent from its function as a COX assembly factor. We propose that dp53 may have a key role in the development of the cardiomyopathy.

CONCLUSIONES

1. La interferencia del factor de ensamblaje *ScoX* de *Drosophila melanogaster* en el corazón, provoca una disfunción mitocondrial acompañada de un cambio metabólico que favorece la glicolisis frente a la fosforilación oxidativa.

2. La interferencia de *ScoX* en el corazón de *Drosophila* causa una severa cardiomiopatía dilatada, además de afectar gravemente a la estructura de las miofibrillas que conforman los cardiomiocitos. Tanto la afección cardiaca como la degeneración observada son dependientes del tiempo.

3. Los defectos estructurales observados en los corazones de los adultos no son debidos a problemas durante el desarrollo.

4. La interferencia de *ScoX* provoca un incremento de las especies reactivas de oxígeno.

5. *dp53* y *ScoX* interactúan genéticamente.

6. *dp53* ejerce un papel esencial en el desarrollo de la cardiomiopatía.

7. La interferencia de *ScoX* provoca la activación de la apoptosis en los cardiomiocitos.

8. La degeneración estructural que se observa en el corazón de *Drosophila* es debida a la activación de la apoptosis y no a la disfunción mitocondrial que causa la interferencia de *ScoX*.

9. La falta de función de Sco2 en el ratón $Sco2^{KIKO}$, provoca la activación de la apoptosis tanto en el hígado como en el músculo esquelético.

10. La proteína SCOX, además de ser un factor de ensamblaje del complejo IV, podría desempeñar una función adicional en la célula.

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